







FACULTEIT DIERGENEESKUNDE  
approved by EAEVE

Vakgroep Virologie, Parasitologie en Immunologie

Laboratorium voor Immunologie

# **Seroprevalence of *Toxoplasma gondii* and immune responses in Belgian sheep**

Delfien Verhelst

Promotoren

Prof. Dr. Eric Cox

Prof. Dr. Pierre Dorny

Proefschrift voorgedragen tot het behalen van de graad van  
Doctor in de Diergeneeskundige Wetenschappen,

Universiteit Gent, 2014

ISBN: 9789058643919

To refer to this thesis:

Verhelst, D. (2014). Seroprevalence of *Toxoplasma gondii* and immune responses in Belgian sheep. Thesis submitted in the fulfillment of the requirements for the degree of Doctor in Veterinary Science (PhD), Faculty of Veterinary Medicine, Ghent University

The author and promoter give permission to consult and to copy parts of this work for personal use only. Every other use is subject to the copy write laws. Permission to reproduce any material should be obtained from the author.

## Table of contents

List of abbreviations .....	5
Part I Review of the literature .....	9
Chapter 1 <i>Toxoplasma gondii</i> .....	11
1.1 History .....	13
1.2 Taxonomy and classification .....	13
1.3 <i>Toxoplasma</i> diversity and subtyping .....	14
1.4 Structures and cell invasion .....	16
1.5 Life cycle .....	21
1.6 References .....	31
Chapter 2 <i>Toxoplasma gondii</i> in sheep .....	37
2.1 Transmission and pathogenesis .....	39
2.2 Immunological responses .....	42
2.3 Diagnostic detection methods .....	43
2.4 Seroprevalence .....	52
2.5 Economic Importance .....	57
2.6 Prevention of infection .....	58
2.7 References .....	62
Part II Aims of the study .....	79
Aims of the study .....	81
Part III Experimental studies .....	83
Chapter 3 Seroprevalence of <i>Toxoplasma gondii</i> in domestic sheep in Belgium .....	85
3.1 Abstract .....	87
3.2 Introduction .....	87
3.3 Material and Methods .....	88
3.4 Results and Discussion .....	90
Acknowledgements .....	93
3.5 References .....	94
Chapter 4 Parasite distribution and associated immune response during the acute phase of <i>Toxoplasma gondii</i> infection in sheep .....	97
4.1 Abstract .....	99
4.2 Introduction .....	100
4.3 Material and Methods .....	101
4.4 Results .....	107
4.5 Discussion .....	118
4.6 Conclusions .....	121
Aknowledgements .....	121
4.7 References .....	123

Chapter 5	Interferon gamma expression and infectivity of <i>Toxoplasma</i> infected tissues from experimentally infected sheep in comparison with pigs .....	129
5.1	Abstract .....	131
5.2	Introduction .....	131
5.3	Materials and Methods .....	133
5.4	Results .....	138
5.5	Discussion .....	151
	Aknowledgements .....	153
5.6	References .....	154
Chapter 6	General discussion .....	157
6.1	Introduction .....	159
6.2	Seroprevalence and risk factors for ovine toxoplasmosis in Belgium .....	159
6.3	The acute and subacute immune response against <i>T. gondii</i> .....	161
6.4	Conclusions .....	168
6.5	References .....	170
	Summary .....	175
	Samenvatting .....	181
	Curriculum vitae .....	189
	Dankwoord .....	195

## List of Abbreviations

AMA1	Apical Membrane Antigen 1
ARSIA	Association régionale de Santé et d'Identification animales
AS	Antisense
β –actin	Beta-actin
BCA	Bicinchoninic acid
C	Control animal
CAE	Caprine Arthritis Encephalitis
CI	Credibility interval
ConA	Concanavalin A
Ct	Threshold value
D	Duodenal
DGZ	Dierengezondheidszorg Vlaanderen / Animal Health Care Flanders
dpi	days post infection
DTT	Dithiothreitol
EC	European Union Council Regulation
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
FASFC	Federal Agency for Safety of Food Chain
FCS	Fetal calf serum
FITC	Fluorescein isothiocyanate
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GRA	Dense granule
H & E	Hematoxylin and Eosin
HBSS	Hank's buffered salt solution
HRP	Horseradish peroxidase
I	Ileal
IFN-gamma	Interferon-gamma
I(I)FA	Indirect Immunofluorescent antibody assay
IHA	Indirect hemagglutination assay
IL	Interleukin
IP	Intraperitoneal
ISAGA	Immunosorbent agglutination assay
ISCOM	Immunostimulating complexes
J	Jejunal
LAT	Latex agglutination test
LD	Lethal dose
LN	lymph node
M.	Musculus
mAb	Monoclonal antibody
MAT	Modified agglutination test
MC	Mononuclear cells
MIC	Microneme proteins
M. gastr	Musculus gastrocnemius
MHC	Major histocompatibility complex
MLE	Multilocus enzyme electrophoresis
MLN	Mesenteric lymph nodes
M. longd	Musculus longissimus dorsi
MS	Microsatellite
MyoA	Myosin A
nd	Not detected
ns	Not specified
OD	Optical density

PAS	Periodic acid Schiff
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PI	Post Infection
PMSF	Phenylmethanesulfonyl fluoride
PP	Peyer's patches
Pr-	Negative agreement index
Pr+	Positive agreement index
PRU	<i>T. gondii</i> Prugniald strain
PV	Parasitophorous vacuole
r	Recombinant
RFLP	Restriction fragment length polymorphism
ROM	Rhomboid
RON	Rhoptry neck protein
ROP	Rhoptry protein
RT	Room temperature
S	Sense
SAG	Surface antigen
Se	Sensitivity
SEM	Scanning electron microscopy
SEM	Standard error of the mean
SFDT	Sabin Feldman dye test
Sh	Sheep
SK-6	Swine kidney cells
SNP	Single nucleotide polymorphisms
Sp	Specificity
Sw	Swine
TEM	Transmission electron microscopy
Th1	T-helper 1 cells
Th2	T-helper 2 cells
TLA	Total lysate antigen
TMB	3,3',5,5'-tetramethylbenzidine
TNF-alfa	Tumor necrosis factor-alfa
VIDA	Veterinary Investigation Diagnosis Analysis







## **PART I: REVIEW OF THE LITERATURE**



## **CHAPTER 1**

***TOXOPLASMA GONDII***



## 1.1 History

*Toxoplasma gondii* is an obligate intracellular parasite that was first described by Charles Nicolle and Louis Manceaux in 1908 in the *Ctenodactylus gundi*, a hamster-like rodent. Nicolle and Manceaux found the protozoan in tissues of a gundi, which was used for Leishmaniasis research in the laboratory of the Pasteur Institute in Tunis. They realised that they had discovered a new organism and named it *Toxoplasma gondii* based on the morphology. The name *Toxoplasma* is based on taxon, which means arc or bow and plasma, which means life and image. *Gondi* may have resulted from a misspelling of the original host the *Ctenodactylus gundi* (Nicolle and Manceaux, 1908, 1909).

In the same year as Nicolle and Manceaux, Splendore discovered the same parasite in Brazil in a rabbit, but he identified it first as *Leishmania* (Ferguson, 2009).

## 1.2 Taxonomy and classification

*T. gondii* is an apicomplexan coccidian parasite. Apicomplexa are intracellular parasites that have a polarised cell structure and a complex of organelles in the apical end (see further). Coccidia are single-celled obligate intracellular parasites that infect the intestinal tract of their hosts. *Toxoplasma gondii* belongs to the family of Sarcocystidae with other members, like *Sarcocystis*, *Neospora*, *Hammondia* and *Besnoitia*.

*Toxoplasma gondii* belongs to (from: <http://www.ncbi.nlm.nih.gov/Taxonomy/>):

Domain: Eukaryota

Kingdom: Alveolata

Phylum: Apicomplexa; Levine, 1970

Class: Coccidia

Subclass: Eucoccidiorida

Order: Eimeriorina

Family: Sarcocystidae

Genus: *Toxoplasma*

Species: *Toxoplasma gondii*

### 1.3 *Toxoplasma* diversity and subtyping

It is known since long that strains of *T. gondii* vary in virulence for mice. Since the development of molecular typing techniques, the genetic variation in *T. gondii* strains could be linked to the phenotypic differences. The first typing experiments showed that mouse virulent strains all belonged to a single clonal genotype, whereas the less virulent ones showed more variability (Sibley, Boothroyd, 1992). Typing more strains showed that the population of *T. gondii* is highly structured and that nearly all strains could be grouped into one of three clonal lineages (type I, II and III) (Darde et al., 1992; Howe, Sibley, 1995; Sibley, Boothroyd, 1992). Direct oral infectivity to other intermediate hosts via tissue cysts may explain their successful expansion (Khan et al., 2006a; Su et al., 2003).

Lots of genotyping methods have been developed. They are usually based on the analysis of polymorphous loci. Their use has indicated a rather unusual population structure.

The first studies on strain typing were based on **multilocus enzyme electrophoresis (MLEE)**, which is an important tool to study genetic relationships. Variations in alleles of housekeeping genes encoding enzymes can be detected by estimating variations in the net electrophoretic charges of the polypeptides, which results in different mobilities and distinct bands when run on a gel. This method has a low resolution all arising from the fact that enzymatic phenotype diversity is merely a proxy for DNA sequence diversity. Polymorphic enzymes were used to characterize *T. gondii* isolates of which most were collected in France. They were grouped into four major zymodemes (patterns of isoenzymes) i.e.: Z1, Z2, Z3 and Z4 (Darde et al., 1992a, 1992b). Despite the specificity of this test it requires a large number of purified parasites, which strengthened the need for new techniques.

Later typing methods were using **microsatellite (MS) markers** (Ajzenberg et al., 2002a, 2002b). MS sequences are tandem repeated segments of short DNA that tend to occur in non-coding DNA. MS markers are sensitive, reliable and amenable to high-throughput analyses; however, the difficulty of this technique is the possible influence of contaminating host DNA, which can be a significant source of variability (Sibley et al., 2009).

Another analysis is based on **restriction fragment length polymorphism (RFLP)** of specific genetic loci. This worldwide used method for genotyping of *T. gondii* is based on a PCR amplification followed by the digestion of the DNA sample by restriction enzymes. The resulting restriction fragments are separated according to their lengths by gel electrophoresis. Isolates, primarily collected from North America and Europe, were characterized using RFLP.



This analysis classified *T. gondii* into three genetic lineages, designated as Type I, Type II and Type III (Ajzenberg et al., 2002a; Ajzenberg et al., 2002b; Howe and Sibley, 1995).

These three genotypes differ less than one percent in their genomic sequence (Sibley and Boothroyd, 1992). However, they differ in virulence in mice. Type I strains (e.g. the RH strain) are lethal in outbred mice: one type I tachyzoite injected intraperitoneally (IP), is able to kill mice (Lethal dose (LD) 100 % = 1). Furthermore, infection with type I strains has been associated with severe or atypical ocular toxoplasmosis in infected immunocompetent adults and they are overrepresented in congenital infections (Grigg and Boothroyd, 2001; Howe and Sibley, 1995). In contrast, type II (e.g. the PRU strain) and III (includes avirulent strains like the VEG strain) strains are less virulent with a LD 100  $\geq 10^3$  tachyzoites IP (Sibley and Boothroyd, 1992). Type II strains account for most clinical toxoplasmosis cases in immunocompromised patients (Grigg and Boothroyd, 2001). Type III strains were found in patients with ocular toxoplasmosis (Fux et al., 2003).

A simplified typing method based on **nested-PCR** of RFLPs at the SAG2 locus, encoding tachyzoite surface antigen P22, was sufficient for characterizing clinical isolates from North America and Europe that belong to the same clonal lineage and have an identical genetic background (Howe et al., 1997). However, this genetic marker has limited power to distinguish parasite isolates and was furthermore inaccurate for recombinant and exotic strains. Many polymorphisms can be missed by RFLP markers because they are based on the variation of restriction enzymes sites (Sibley et al., 2009; Su et al., 2006).

All of the above methods underestimate the true rate of polymorphism. In contrast, **direct sequencing** reveals the complete genetic diverseness such as single nucleotide polymorphisms (SNPs), small insertions and deletions (Bontell et al., 2009; Frazao-Teixeira et al., 2011). This method demonstrated a much larger genetic diversity in *T. gondii*, which forms an advantage in the typing of new discovered exotic strains (Frazao-Teixeira et al., 2011). Increased costs and the need for sophisticated technology are the major drawbacks of this technique, but on the other hand, more cost-effective typing methods could be developed to detect alleles, once the population structure for a given region is known.

A **15-MS multiplex assay**, was developed by Ajzenberg et al. (2010), to compare two isolates and to determine if they are genetically identical or not. This can be useful for the determination of the common source of infection in an outbreak or to trace laboratory contamination or to reveal mixed infections. A first genetic screening should rapidly distinguish type II, III or atypical strains. A second level of discrimination is the fingerprinting level, to discriminate closely related strains belonging to the same lineage. The

15-MS multiplex assay is a rapid genotyping method which aims to ensure both levels of genetic discrimination of *T. gondii* isolates in a single PCR assay using 15 MS markers located on 11 different chromosomes of the parasite (Ajzenberg et al., 2010).

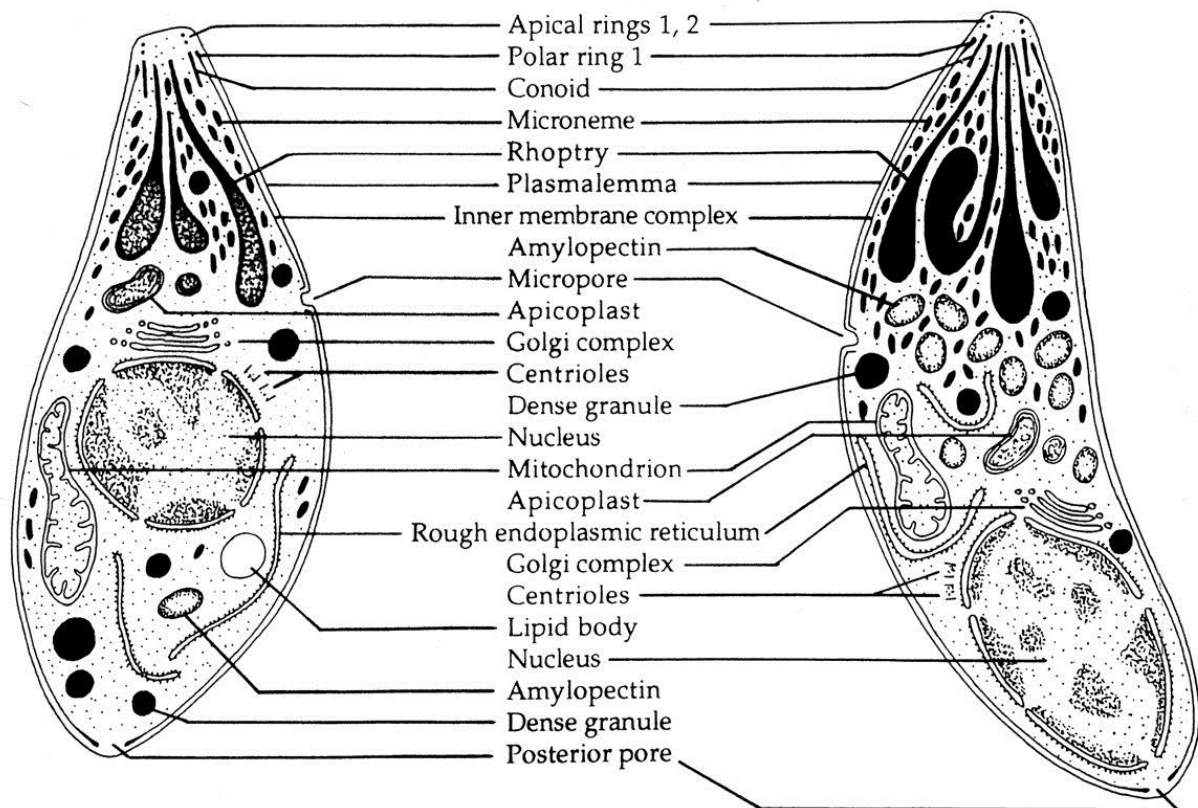
## **1.4 Structures and cell invasion**

*T. gondii* occurs in three infectious stages i.e.: tachyzoites, which are responsible for the spread during acute infection, bradyzoites, which sustain the chronic infection and sporozoites within the oocysts, which disseminate in the environment. These three stages are haploid; tachy- and bradyzoites show an asexual division. The sexual cycle occurs in the enterocytes of cat intestines with the formation of diploid oocysts, which form the sporozoites as a result of meiosis. The role of bradyzoites and sporozoites is to transmit infection between hosts (Ferguson, 2004). A tachyzoite and bradyzoite are represented in Figure 1.1.

### **1.4.1 Tachyzoites**

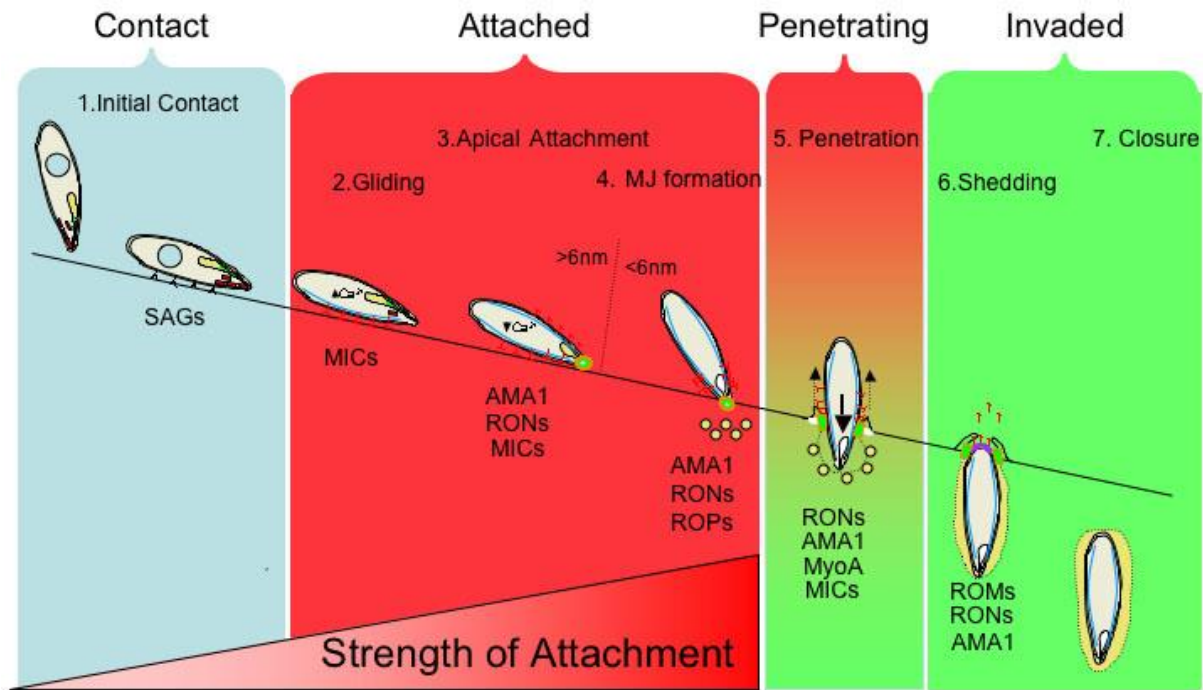
The tachyzoite is the rapidly multiplying stage of the parasite. It is crescent shaped with a funnel shaped anterior end and a convex posterior end. The size of a tachyzoite is about 2 x 6  $\mu\text{m}$ . It has a complex structure with various organelles and inclusion bodies.

The tachyzoites can infect and replicate in all mammalian and avian cells except in mammalian red blood cells. The infection of a host cell consists of the following steps (Figure 1.2): after the identification of a host cell, the tachyzoite moves towards the cell by gliding, flexing, undulating or rotating. The tachyzoite glides over the cell membrane, probes the host cell with the conoidal (a small cone-shaped structure comprised of a spiral of novel filaments), followed by indenting the plasmalemma of the host cell. The initial binding between the parasite and the host cell is mediated by surface antigen 1 (SAG-1). Next, the microneme proteins connect the apical end with the host cell surface. SAG-2 is involved in the apical orientation, which is essential for invasion (Nichols et al., 1983).



**Figure 1.1:** Schematic drawing of a tachyzoite (left) and a bradyzoite (right) of *T. gondii*. The drawings are composites of electron micrographs. Reprinted from Dubey et al. (1998) with permission of the publisher.

The next step is the formation of the moving junction. This moves along the tachyzoite during his penetration into the host cell. The whole procedure is mediated by  $\text{Ca}^{2+}$  dependent events of the conoid protrusion (Bonhomme et al., 1992).



**Figure 1.2:** Conceptual model of *Toxoplasma* invasion.

Adapted from: <http://www.biostat.jhsp.edu/bkafsack/Bioinformatics/>

1. Cellular recognition and initial contact with the host cell membrane mediated by Surface Antigens (SAG).
2. Gliding of the parasite along the host cell and release of microneme proteins (MIC).
3. Once there is an apical attachment, rhoptry neck proteins (RON) and apical membrane antigen 1 (AMA 1) form a multimeric complex for the apical orientation.
4. Formation of a moving junction between tachyzoite membrane and the host cell (Besteiro et al., 2011).
5. - 7. Penetration into the host cell results in rhoptry protein and myosin A (MyoA) secretion and formation of the parasitophorous vacuole (PV), with the secretion of the rombooids (ROMs) (Besteiro et al., 2011).

During the formation of moving junctions, microneme proteins (MIC), rhoptry proteins (Besteiro et al., 2011) and dense granules (GRA) are released. The microneme secretion is rapidly up-regulated when *T. gondii* makes contact with the host cell, which confirms its role in motility, migration and invasion (Carruthers and Tomley, 2008). Furthermore the parasite invasion is blocked when the microneme secretion is blocked (Carruthers et al., 1999). Once the penetration of the conoid starts, the parasitophorous vacuole (PV) is formed by the secretion of rhoptry proteins (ROPs) (Besteiro et al., 2011). Dense granule-associated proteins (GRA-associated) are secreted once the parasite resides in the parasitophorous vacuole and are present in the parasitophorous vacuolar space and in the parasitophorous vacuole

membrane. GRA proteins promote the maturation of the parasitophorous vacuole and optimize the cellular environment to fit the requirements for growth and persistence of the parasite in host tissues (Bougdour et al., 2013; Carruthers and Sibley, 1997). They mediate the formation of a tubular intravacuolar network for exchanges between the host cell cytoplasm and the parasitophorous vacuole. Sibley et al. (1995) describe the role of this membranous nanotubular network as to establish a metabolic connection with the host cell. Once this is accomplished, the tachyzoites begin to divide asexually in the parasitophorous vacuole by endodyogeny, a mode of reproduction characterized by the synchronous production of two daughter progenies within each mother cell (Morrisette and Sibley, 2002; Nishi et al., 2008). Tachyzoites replicate inside a cell with a generation time of 6 to 9 h until egress, usually after 64 to 128 parasites have accumulated (Radke and White, 1998). A variety of factors such as, the growth of the tachyzoites, the death receptor ligation in infected cells and micro-environmental changes, due to the host immune response (interferon-gamma, nitric oxide), lead to the rupture of the host cell whereafter the tachyzoites infect new neighbouring host cells (Ferguson, 2004; Ji et al., 2013). The exit is mediated by the parasite secreted *T. gondii* perforin-like protein 1 (TgPLP1) (Kafsack et al., 2009).

The rates of invasion and growth depend on the *T. gondii* strain and the host cells. After entry of tachyzoites into a host cell, there is a variable lag phase before the parasite starts endodyogeny, and this lag phase is partly strain dependent (Appleford and Smith, 1997; Dubey, 2010). The virulent RH strain has a shorter lag phase than the mouse virulent strains ENT and Martin, but, the ENG strain tachyzoites divide more rapidly than RH tachyzoites, although they all belong to the clonal lineage type I (Appleford and Smith, 1997).

The tachyzoites undergo stage conversion to a semidormant stage, known as bradyzoites, settled in tissue cysts, due to environmental stress and the host immune response (Sibley et al., 2009).

### **1.4.2 Bradyzoites**

The bradyzoite is an encysted, slowly dividing stage of the parasite. The size is approximately 5-8.5 x 1-3 µm. Young tissue cysts contain only two bradyzoites and measure barely 5 µm while older ones can contain thousands of bradyzoites formed by endodyogeny (Dubey, 2010). Cyst-like structures appear as early as three days after infection with tachyzoites, and become numerous by day 6 (Dubey and Frenkel, 1976). Although tissue cysts appear in visceral organs such as lungs, liver and kidneys, they are found predominantly

in the central nervous system and in muscle tissue, where they may stay for the host's life (Black and Boothroyd, 2000). The development of tissue cysts throughout the body defines the chronic stage of the asexual cycle. The conversion of bradyzoites to tachyzoites and vice versa is important for *T. gondii*'s life cycle (Dubey, 2006). The trigger of the *T. gondii* encystation and the molecular mechanisms for the switch from tachyzoite to bradyzoite remain unknown. During the transformation to bradyzoites, *T. gondii* modifies its morphology and possibly its metabolism to adjust to the environmental changes (Tomavo, 2001).

The infectivity of bradyzoites differs from one animal to animal. Cats can shed millions of oocysts after ingesting only one bradyzoite, whereas 100 bradyzoites may not be enough to infect mice by the oral route (Dubey, 2001).

Upon entering a new host cell, bradyzoites appear unable to produce new generations of bradyzoites or sporozoites but default immediately to tachyzoite development (Dubey, 1997).

### ***1.4.3 Sporozoites within oocysts***

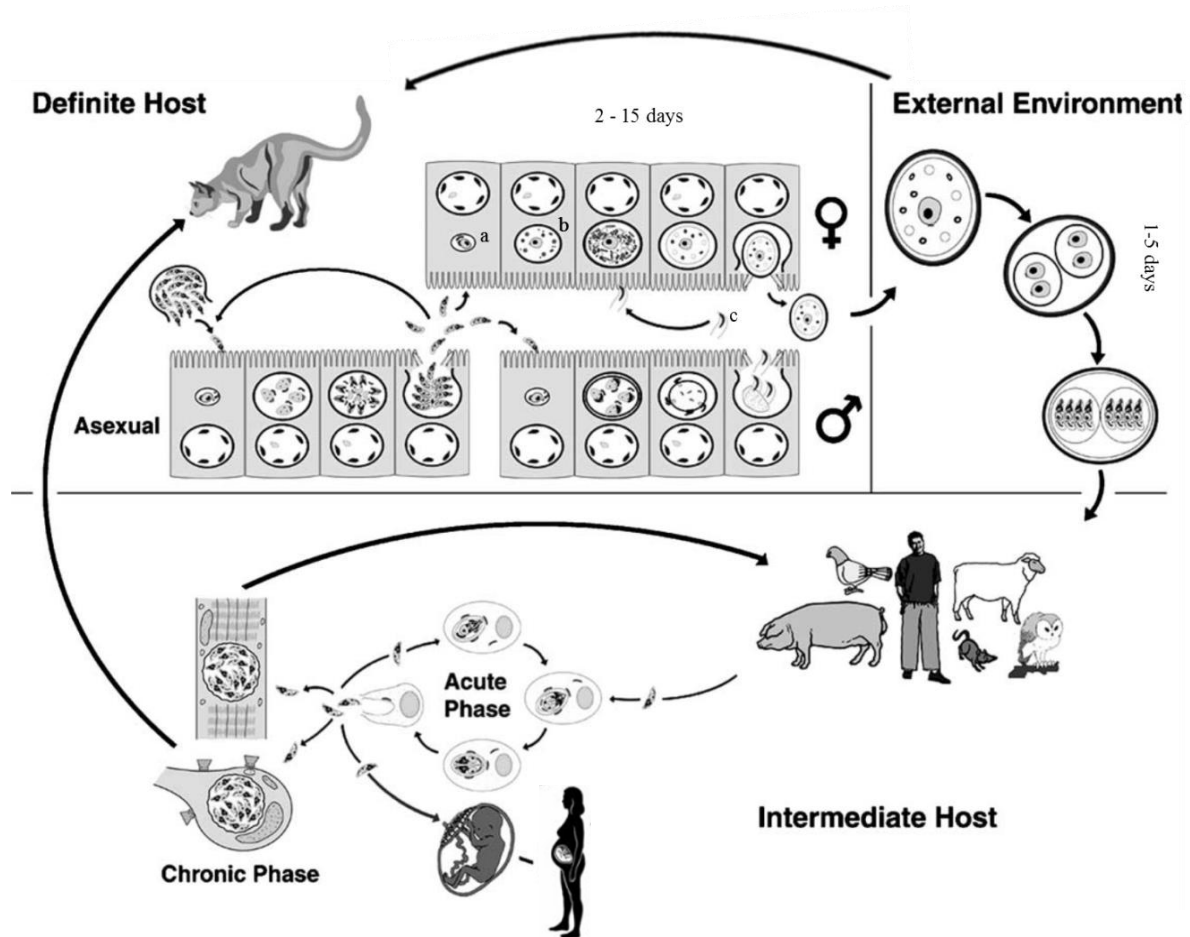
Sporozoites are present in sporulated oocysts. Only domestic and wild Felidae shed oocysts in their faeces. The sporulation takes place in the environment within 1 to 5 days after shedding, depending on temperature and humidity. Each sporulated oocyst contains two ellipsoidal sporocysts and each sporocyst contains four sporozoites See 1.5.1 "Life cycle in definitive host, the cat". Oocysts become infectious after sporulation and can remain infectious for several months under favourable conditions (Dubey, 1998).

## 1.5 Life cycle

Splendore wrote in 1908: “I think that it will be impossible to determine a specific classification of this new protozoa(n) before the whole life cycle is known, and I hope to discover this in my future research....” (Splendore, 1908). The elucidation of the life cycle was indeed essential to establish that *T. gondii* is a coccidian parasite. Unfortunately, Splendore was not able to clarify the whole cycle, but he discovered the basics of the parasite’s life cycle. The final resolution of the *T. gondii* life cycle took place approximately 60 years after his prophetic words, with the discovery in the late 1960s of the central role of the cat as a definitive host in spreading oocysts through its faeces (Robert-Gangneux and Darde, 2012). All related species of the Apicomplexa have similar complex two-host life cycles that alternate between definitive (sexual propagation) and intermediate hosts (asexual replication). *Toxoplasma gondii* has a remarkable extremely wide range of intermediate hosts that includes all birds and mammals. Sexual propagation is limited to members of the cat family (Felidae) (Black and Boothroyd, 2000). The *T. gondii* life cycle consists of multiple stages that fluctuate between proliferative and latent. As described before, there are three different infectious stages of *T. gondii*: the tachyzoites, responsible for the acute infection; the bradyzoites, responsible for the chronic infection, and the sporozoites within the oocysts (Dubey, 1998).

### ***1.5.1 Life cycle in the definitive host, the cat and other Felidae.***

Felidae, including domestic cats, can shed oocysts after a *T. gondii* infection with any of the three infectious stages. The prepatent period (time to the shedding of oocysts after initial infection) and the amount of oocyst shedding depend on the stage of the ingested parasites varying from 3 to 18 days after ingestion and is not dependent of the dose. Cats with a *T. gondii* primary infection can shed up to  $10^6$  oocysts per day with their faeces for a period of two weeks at most. Less than 50% of the cats shed oocysts after ingesting tachyzoites or oocysts, whereas nearly all cats shed oocysts after ingestion of tissue cysts.

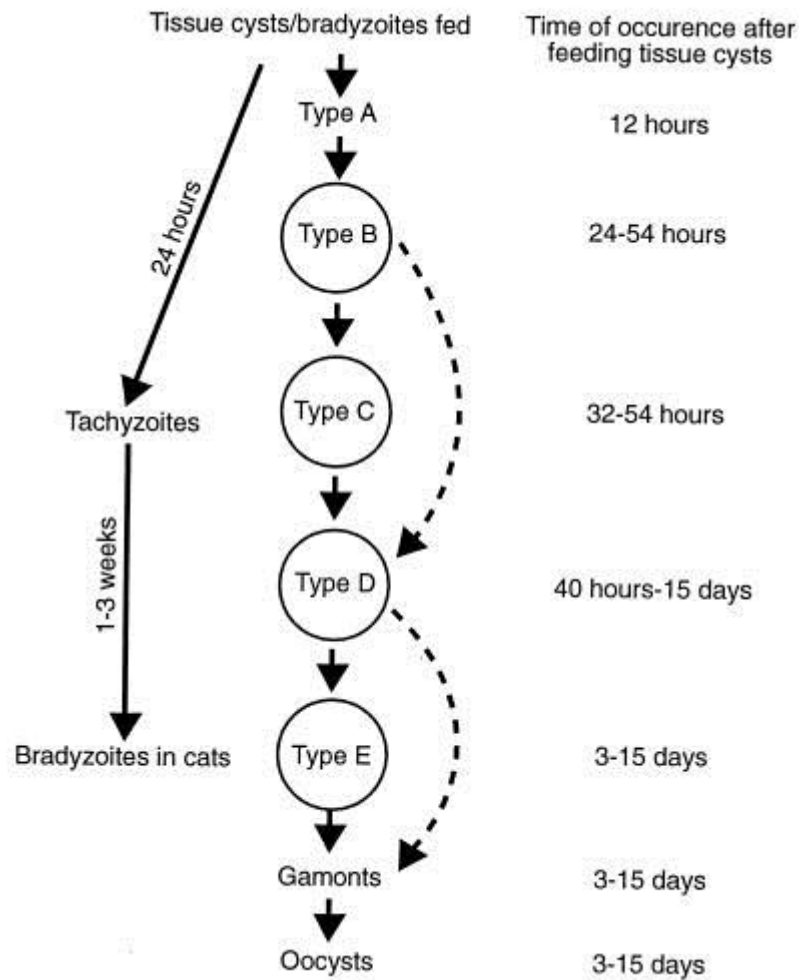


**Figure 1.3:** Schematic representation of the life cycle of *T. gondii*.

a: macrogamont; b: macrogamete; c: microgamete. Adapted from Ferguson (2004).

After ingestion of tissue cysts or oocysts, the wall of the tissue cysts or oocysts is digested by the proteolytic enzymes secreted in the stomach and the small intestine. The released bradyzoites/sporozoites penetrate the epithelial cell of the small intestine where they differentiate into the rapidly dividing tachyzoite stage during the enteroepithelial cycle with the development of numerous generations of *T. gondii* for dissemination throughout the body (Figure 1.3). After a few asexual generations in the feline small intestinal epithelial cells, five morphologically different stages of the parasite develop by schizogony followed by gametogony. Each stage (schizont A to E) will multiply during several generations in the epithelium (Figure 1.4) (Dubey, 1998; Dubey and Frenkel, 1972). The sexual cycle starts two days after tissue cyst ingestion by a Felidae. How gamonts exactly appear has not yet been determined. The gamete formation is probably induced by merozoites (not in Figure 1.4) released by schizont stages D and E.





**Figure 1.4:** Coccidian cycle of *T. gondii*. Reprinted from Dubey and Frenkel (1972) with permission of the publisher.

Three to 15 days after ingestion, micro- (male) and macro- (female) gamonts appear in the small intestines, mostly in the ileum (Dubey et al., 1998). The macrogamonts will mature into macrogametes. During the microgametogenesis, microgametes are produced by several nuclear divisions. Microgametes have 2 flagellae that make them able to swim to the macrogametes. A mature female and male macrogamete fertilise to a zygote, which becomes surrounded by a thick wall. The oocyst is formed. On maturity, infected epithelial cells rupture and discharge oocysts in the intestinal content and subsequently via the faeces into the environment (Ferguson et al., 1979a). The prepatent period (time to the shedding of oocysts after initial infection) varies from 3 to 10 days after the ingestion of tissue cysts, and can be as long as 18 days after ingestion of oocysts or tachyzoites.

Once outside the body, sporulation of oocysts takes place within 1 to 5 days, depending on temperature and humidity (Dubey et al., 2011; Dubey and Frenkel, 1972; Ferguson et al., 1979b, c). During sporulation, the 2 sporoblasts, each with two nuclei, elongate and two

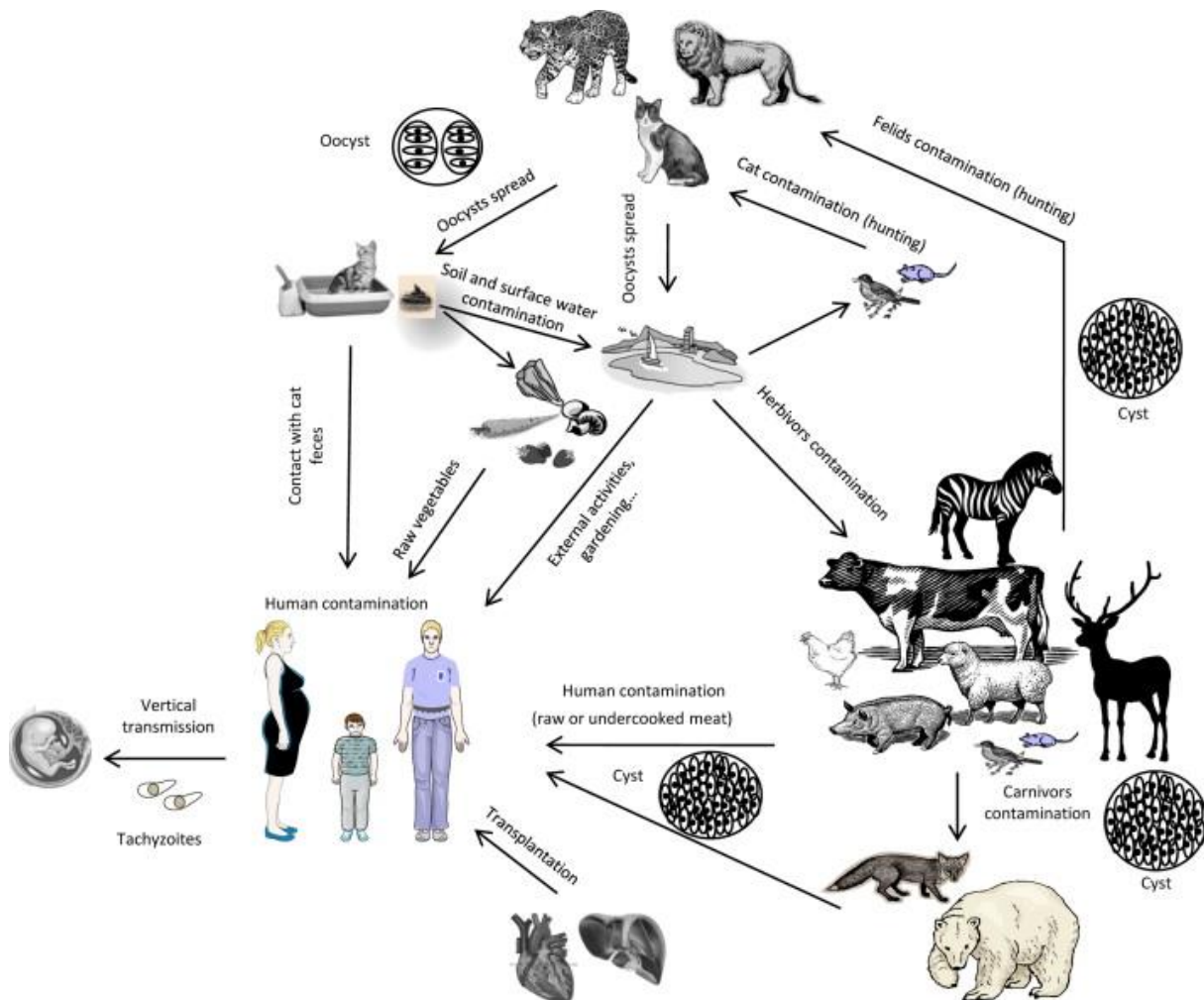
ellipsoidal sporocysts are formed (Figure 1.3). The sporozoite formation begins when two dense plaques or analagen appear at both ends of the sporocysts. Each nucleus divides into two and is incorporated into the elongating sporozoite analagen (Dubey and Frenkel, 1972; Ferguson et al., 1979c). Oocysts are highly infectious after maturation. When oocysts are ingested by a cat, the cycle can start again. Sporulated oocysts can survive for several months in moist conditions.

### ***1.5.2 Life cycle in the intermediate hosts, including humans and cats***

The asexual phase can take place in humans and each warm-blooded animal, including cats, acting as intermediate hosts (Wong and Remington, 1993). After ingestion, the sporozoites, present in the oocysts, or the bradyzoites, in tissue cysts will infect the intestines and, as already mentioned for Felidae, undergo stage conversion to tachyzoites. In contrast to Felidae, no differentiation to schizonts occurs in other species. The tachyzoites disseminate through the body during the acute phase of the infection and convert into bradyzoites encapsulated in tissue cysts (Dubey et al., 1998). When infectious tissue cysts, present in raw or undercooked meat are ingested, their wall is dissolved by proteolytic enzymes in the stomach and the small intestine. As previously described, the released bradyzoites penetrate the small intestinal wall and finalise the asexual cycle.

In our literature review we only discuss the situation in humans and in sheep and pigs, because it is mainly in these hosts that *T. gondii* infection causes clinical symptoms and reproductive disorders. Furthermore, pork and mutton are considered as an important source of human toxoplasmosis. Although the seroprevalence in pigs is low, pork can still be considered as a source for human toxoplasmosis as meat of one pig is eaten on average by 300 consumers and is used in many meat preparations (Fehlhaber et al., 2002).

Ovine toxoplasmosis is described in the second chapter of this thesis.



**Figure 1.5:** Sources of human *Toxoplasma* infection. Reprinted from Robert-Gangneux (2014) with permission of the publisher.

### 1.5.2.1 *Toxoplasma gondii* infection in humans

#### 1.5.2.1.1 Clinical aspects of toxoplasmosis

*Toxoplasma gondii* is capable of producing a wide range of symptoms. Clinical signs are depending on the ability of the host immune system to limit the spread, and on the number of released parasites. In most immunocompetent persons the cell-mediated immune response can control the infection, so that it usually passes unnoticed (Gazzinelli et al., 1993). Sometimes, flu-like symptoms develop such as fever, swollen lymph nodes, muscle aches,... , but it may take weeks or months before symptoms of infection appear, if they appear at all and this even though the parasites grow and multiply immediately after entering the body.

It is thought that once someone becomes infected with *T. gondii*, the infection remains in the body for life, usually in a latent (inactive) form that won't cause side effects or harm. The infection can reactivate when the immune system becomes compromised, e.g. by HIV infection, cancer and/or immunosuppressive therapies. In humans with a weakened immune system, Toxoplasmosis can even be fatal.

*Toxoplasma* has also a clinical impact on the unborn foetus (Dubey, 2010). When a pregnant woman acquires toxoplasmosis during pregnancy - even without symptoms - and remains untreated, there is a chance that she will pass the infection on to the developing foetus (Hay and Hutchison, 1983). Successful pregnancy requires the maternal host to develop a balance between a tolerance to the semi-allogenic foetus and the immune reactivity to protect the mother against infections. The exact mechanisms for the tolerance remains poorly understood (Alijotas-Reig et al., 2014). A T-helper 1 (Th1)/Th2 response at the foeto-maternal interface is required. During pregnancy, placental cells invade the uterine wall to form cotyledons. This stimulates CD4+ and CD8+ lymphocytes, macrophages and plasma cells. Despite these responses, the pregnancy is unaffected. It is possible that these interactions lead to a Th2 immune response, dominated by IL-10 production, rather than a Th1 response that could lead to rejecting of the foetus. However, down-modulation of the Th1 response means an increase in maternal susceptibility to *T. gondii* and, in the meantime a down-modulation of the mechanism that controls the parasite replication and promotes congenital transmission due to a decreased production of IFN-gamma and a depletion of NK cells (Krishnan et al., 2013). Kravetz (2013) mentioned that about five percent of non-immune pregnant women may acquire toxoplasmosis, with a ten to 100 % risk of vertical transmission to the unborn child. This indicates that a normal functioning intact immune system is absolutely essential for controlling the parasite.

Unborn children who become infected during their mother's first trimester tend to have the most severe symptoms of congenital toxoplasmosis. In other words, the risks of transmission to the baby are higher later in pregnancy, but the risks for lesions to the foetus as a consequence of infection are greater earlier in pregnancy (Kravetz, 2013). Women who acquire the infection before pregnancy will rarely pass the infection on to their unborn baby because they will have built up immunity bringing the infection to latency. However, if a pregnant woman, who had been infected previously, becomes immunocompromised by therapy or by an infection, the parasite can become reactivated (Tenter et al., 2000). Up to 90 % of children born with congenital toxoplasmosis have no clinical signs early in infancy, but a large percentage will show signs of infection months to years later. Apart from early

death after birth, long-term symptoms may develop such as, mental or motoric retardation, hydroencephalitis, jaundice, anaemia, enlarged liver and/or spleen, hearing loss,... (Fayer, 1981). The few babies who show clear signs of infection at birth or shortly after birth may be born prematurely and are unusually small at birth. Congenital infected children are also at high risk for eye damage involving the retina, resulting in severe vision problems, which may appear in early childhood or during adolescence (Kijlstra and Petersen, 2014).

#### 1.5.2.1.2 Epidemiology

In a study done in 2008, toxoplasmosis appeared as the fifth most important foodborne zoonotic pathogen in Belgium after *Salmonella* spp., *Campylobacter* spp., *Listeria monocytogenes* and verocytotoxic *Escherichia coli* (FAVV, 2008). Furthermore, in the United States there is a yearly incidence of 3000 human cases; the associated economic impact is estimated at €5.98 billion per year (Buzby and Roberts, 1997), representing the sum of the medical costs, special education and residential-care costs, and the social costs due to unemployment and the lost productivity.

The European Food Safety Authority (EFSA) recommended in 2007 that, in order to better evaluate the disease risk of toxoplasmosis in member states, there is a need to improve data collection on surveillance and monitoring of *T. gondii* in animals and food products for human consumption (EFSA, 2007). The seroprevalence of *T. gondii* in humans is approximately 50% in Belgium (Vanhaesebrouck et al., 2003). In Scandinavian countries, Great Britain, the US and Canada, the seroprevalence is only 10 to 15 % (Jones et al., 2001). Possible reasons for this lower prevalence are other culinary habits, consumption of meat from “indoor farmed” animals and more frequent consumption of frozen meat (Cook et al., 2000; Foulon et al., 1994). The frequency of maternal seroconversion in the EU is two (e.g. Norway) to eight (e.g. France, Belgium, Austria) per 1,000 pregnancies. The overall risk of foetal *T. gondii* transmission during maternal infection is 20-30 %. This risk increases significantly with increasing gestational age. Congenital infection during the first five weeks after conception is very rare (Emna et al., 2006; Press et al., 2005).

The incidence of congenital toxoplasmosis in Belgium is 5 to 10 per 10,000 births (W Foulon, personal communication). Eating insufficiently cooked meat (mostly non-frozen sheep and outdoor raised pork) that contains tissue cysts, is the main source of *T. gondii* infection for pregnant woman. Between 30% and 63% of the infections during pregnancy were attributed to consumption of undercooked or cured meat products in a European

multicentric case-control study (Cook et al., 2000). Contact with soil contaminated by oocysts, e.g. unwashed vegetables or fruits, is the second most important source. Direct contact with cats is not very important in terms of transferring the infection (Cook et al., 2000).

There is a seasonal variation in human toxoplasmosis in many areas of the world. In Slovenia, the incidence of acute toxoplasmosis was significantly higher ( $p < 0.025$ ) during winter and spring than during summer and autumn, and in pregnant women significantly higher ( $p < 0.01$ ) during winter than in summer (Logar et al., 2005). This seasonal pattern occurs also in Austria where a twofold increase in seroconversion was found during winter months compared to summer months (Sagel et al., 2010). A retrospective study in the Netherlands investigating the birth months of 532 patients with ocular toxoplasmosis showed a significantly higher incidence in individuals born in May and a significantly lower incidence in those born in November (Meenken et al., 1991). Other studies reported the seasonality of receiving samples for *T. gondii* testing. A UK study reported a peak from November to February, and a decrease in September (Bannister, 1982), but in another UK study no seasonal pattern was reported (Ryan et al., 1995). In Canada, reports on toxoplasmosis were relatively evenly distributed during the year except for September-November, when there was a decline (Tizard et al., 1976). In Serbia, acute infections occurred more often between October and March ( $p = 0.05$ ) (Bobic et al., 2010). Taking all data together, it appears that human *T. gondii* infections are more common in winter and show a drop in the fall months. One of the hypotheses is that during colder months there is a more frequent and closer contact with cats that are potentially *Toxoplasma*-infected. Indeed cats prefer to spend more time indoors during the winter so that there is an increased risk that cats will be excreting oocysts in the house (Logar et al., 2005).

#### ***1.5.2.2 Toxoplasma gondii infection in pigs***

*T. gondii* infection in pigs has a worldwide distribution (Dubey, 2009b). Normally, pigs used for meat consumption are raised indoors in well-managed facilities on a concrete bedding, and are given regular pig feed. While open production systems improve animal welfare, they increase food safety risks due to bacterial, viral or parasitic infections or even environmental contaminants (Kijlstra et al., 2009; Schoeters and Hoogenboom, 2006; Van Overmeire et al., 2006). There is a difference between organic and "free-range pigs" (Kijlstra et al., 2004). Free-range pigs have outdoor access, straw bedding and are fed with regular pig

feed, whereas organic pigs are reared outdoor for at least 80% of their live. Organic pig raising is controlled by the European Union Council Regulation (EC) No 834/2007 of 28 June 2007 on organic production, labelling of organic products and repealing regulation (EEC) No 2092/91. The EEC implicates outdoor access, organic pig feed, no feed of “animal” origin, more comfortable living space than on intensive farms, weaning at later age, restricted antibiotic and drugs use. Organic pig feed contains the same plant ingredients as regular pig feed, but is grown on farms where the use of chemical fertilisers or pesticides is forbidden. One treatment of synthetic drugs or antibiotics is allowed in organic slaughter pigs during their life. When more treatments are needed the pigs must be sold as cheaper regular pigs (Kijlstra et al., 2004).

During the 90s the *T. gondii* seroprevalence in pigs under intensive management was decreasing in some European countries; however, since then there is a trend for breeding pigs with outdoor access, resulting again in an increasing seroprevalence (Kijlstra et al., 2004; Meerburg et al., 2006). Toxoplasmosis in pigs seldom has a clinical outcome, but mortality can occur, especially in neonatal piglets. Nevertheless, in 2001 on a small pig farm in Thailand, seventeen-day-old piglets had fever, convulsions, dyspnoea, diarrhoea and some died as a result of toxoplasmosis. The morbidity and mortality rates were 26.09 % (6/23) and 4.35 % (1/23), respectively. Histopathology showed interstitial pneumonia and smears of the lungs contained tachyzoites of *T. gondii*. The latex agglutination test on serum samples confirmed the diagnosis of toxoplasmosis. Two possible routes of transmission were suggested: transplacental and the oral infection route via ingestion of oocyst contaminated feed (Thiptara et al., 2006). Another acute *Toxoplasma* outbreak of abortion with mortality of sows was reported in 2002 in Korea (Kim et al., 2009). The clinical signs in the pregnant sows were fever, vomiting, anorexia, depression, recumbency, prostration, abortion and death. The abortion rate was 44 % and the mortality rate of the sows was 19 %. On necropsy, cutaneous cyanosis, enlarged lymph nodes, hepatomegaly and splenomegaly, necrotizing pneumonia, multiple necrotic foci in the liver, spleen, and lymph nodes were found. *T. gondii* tachyzoites were seen by microscopic examination of the lesions. No significant gross abnormalities were noted in any of the aborted foetuses. In the aborted sows, high antibody titers to *T. gondii* were measured. One month before the disease outbreak, the source of commercial feed was changed, but the feed was not tested, therefore, the source of this outbreak remained unknown. In 2004, a severe outbreak occurred on a farm in the Gansu Province in China. During this outbreak, 594 out of 960 fattening pigs became ill. The clinical signs were fever up to 40-42°C, loss of appetite and depression. Antibiotic treatment of

penicillin and streptomycin was without effect. Blood samples were examined by hemagglutination inhibition assay for *T. gondii* antibody detection. Although all the ill pigs were treated with trimethoprim and sulfamethoxazole and their feed was changed, the *T. gondii* infection still resulted in the death of 19 pigs. Feed contaminated with cat faeces was found to be the source of the infection (Li et al., 2010). In none of these cases, genotyping of the parasite was done.

The major risk factors for toxoplasmosis in pigs are contaminated feed, cat access and the presence of seropositive rodents (Kijlstra et al., 2008; Meerburg et al., 2006). Other risk factors are the origin of pigs (farrow-to-finish farming types compared to finishing or farrow-to-breed farming types), age, number of pigs on a farm and carcass disposal methods (Hill, 2010; Villari et al., 2009).



## 1.6 References

- Ajzenberg, D., Banuls, A.L., Tibayrenc, M., Darde, M.L., 2002a. Microsatellite analysis of *Toxoplasma gondii* shows considerable polymorphism structured into two main clonal groups. *Int J Parasitol* 32, 27-38.
- Ajzenberg, D., Cogne, N., Paris, L., Bessieres, M.H., Thulliez, P., Filisetti, D., Pelloux, H., Marty, P., Darde, M.L., 2002b. Genotype of 86 *Toxoplasma gondii* isolates associated with human congenital toxoplasmosis, and correlation with clinical findings. *J Infect Dis* 186, 684-689.
- Ajzenberg, D., Collinet, F., Mercier, A., Vignoles, P., Darde, M.L., 2010. Genotyping of *Toxoplasma gondii* isolates with 15 microsatellite markers in a single multiplex PCR assay. *J Clin Microbiol* 48, 4641-4645.
- Alijotas-Reig, J., Llurba, E., Gris, J.M., 2014. Potentiating maternal immune tolerance in pregnancy: a new challenging role for regulatory T cells. *Placenta* 35, 241-248.
- Appleford, P.J., Smith, J.E., 1997. *Toxoplasma gondii*: the growth characteristics of three virulent strains. *Acta tropica* 65, 97-104.
- Bannister, B.A., 1982. Infectious diseases. *Nursing (Lond)* 2, 195-202.
- Besteiro, S., Dubremetz, J.F., Lebrun, M., 2011. The moving junction of apicomplexan parasites: a key structure for invasion. *Cell Microbiol* 13, 797-805.
- Black, M.W., Boothroyd, J.C., 2000. Lytic cycle of *Toxoplasma gondii*. *Microbiol Mol Biol Rev* 64, 607-623.
- Bobic, B., Klun, I., Nikolic, A., Vujanic, M., Zivkovic, T., Ilovic, V., Djurkovic-Djakovic, O., 2010. Seasonal variations in human *Toxoplasma* infection in Serbia. *Vector Borne Zoonotic Dis* 10, 465-469.
- Bonhomme, A., Pingret, L., Pinon, J.M., 1992. Review: *Toxoplasma gondii* cellular invasion. *Parassitologia* 34, 31-43.
- Bontell, I.L., Hall, N., Ashelford, K.E., Dubey, J.P., Boyle, J.P., Lindh, J., Smith, J.E., 2009. Whole genome sequencing of a natural recombinant *Toxoplasma gondii* strain reveals chromosome sorting and local allelic variants. *Genome Biol* 10, R53.
- Bougdour, A., Tardieux, I., Hakimi, M.A., 2013. *Toxoplasma* exports dense granule proteins beyond the vacuole to the host cell nucleus and rewires the host genome expression. *Cell Microbiol*.
- Buzby, J.C., Roberts, T., 1997. Economic costs and trade impacts of microbial foodborne illness. *World health statistics quarterly. Rapport trimestriel de statistiques sanitaires mondiales* 50, 57-66.
- Carruthers, V.B., Giddings, O.K., Sibley, L.D., 1999. Secretion of micronemal proteins is associated with *toxoplasma* invasion of host cells. *Cell Microbiol* 1, 225-235.
- Carruthers, V.B., Sibley, L.D., 1997. Sequential protein secretion from three distinct organelles of *Toxoplasma gondii* accompanies invasion of human fibroblasts. *Eur J Cell Biol* 73, 114-123.

- Carruthers, V.B., Tomley, F.M., 2008. Microneme proteins in apicomplexans. *Sub-cellular biochemistry* 47, 33-45.
- Cook, A.J., Gilbert, R.E., Buffolano, W., Zufferey, J., Petersen, E., Jenum, P.A., Foulon, W., Semprini, A.E., Dunn, D.T., 2000. Sources of *Toxoplasma* infection in pregnant women: European multicentre case-control study. European Research Network on Congenital Toxoplasmosis. *BMJ* 321, 142-147.
- Darde, M.L., Bouteille, B., Pestre-Alexandre, M., 1992a. Isoenzyme analysis of 35 *Toxoplasma gondii* isolates and the biological and epidemiological implications. *J Parasitol* 78, 786-794.
- Darde, M.L., Riahi, H., Bouteille, B., Pestre-Alexandre, M., 1992b. Isoenzyme analysis of *Hammondia hammondi* and *Toxoplasma gondii* sporozoites. *J Parasitol* 78, 731-734.
- Dubey, J.P., 1997. Tissue cyst tropism in *Toxoplasma gondii*: a comparison of tissue cyst formation in organs of cats, and rodents fed oocysts. *Parasitology* 115 ( Pt 1), 15-20.
- Dubey, J.P., 1998. Advances in the life cycle of *Toxoplasma gondii*. *Int J Parasitol* 28, 1019-1024.
- Dubey, J.P., 2001. Oocyst shedding by cats fed isolated bradyzoites and comparison of infectivity of bradyzoites of the VEG strain *Toxoplasma gondii* to cats and mice. *J Parasitol* 87, 215-219.
- Dubey, J.P., 2006. Comparative infectivity of oocysts and bradyzoites of *Toxoplasma gondii* for intermediate (mice) and definitive (cats) hosts. *Vet Parasitol* 140, 69-75.
- Dubey, J.P., 2009. Toxoplasmosis in pigs--the last 20 years. *Vet Parasitol* 164, 89-103.
- Dubey, J.P., 2010. *Toxoplasmosis of Animals and Humans*. (2nd ed.) CRC Press, Boca Raton, FL 33487, USA, 313 p.
- Dubey, J.P., Ferreira, L.R., Martins, J., Jones, J.L., 2011. Sporulation and survival of *Toxoplasma gondii* oocysts in different types of commercial cat litter. *J Parasitol* 97, 751-754.
- Dubey, J.P., Frenkel, J.K., 1972. Cyst-induced toxoplasmosis in cats. *J Protozool* 19, 155-177.
- Dubey, J.P., Frenkel, J.K., 1976. Feline toxoplasmosis from acutely infected mice and the development of *Toxoplasma* cysts. *J Protozool* 23, 537-546.
- Dubey, J.P., Lindsay, D.S., Speer, C.A., 1998. Structures of *Toxoplasma gondii* tachyzoites, bradyzoites, and sporozoites and biology and development of tissue cysts. *Clin Microbiol Rev* 11, 267-299.
- EFSA, 2007. Scientific opinion of the panel on Biological Hazards and Animal Health Animal Welfare on a request from the European Food Safety Authority (self mandate) to issue a scientific opinion on the review of the Community Summary Report on Trends and Sources of Zoonoses, Zoonotic Agents and Antimicrobial Resistance in the European Union in 2005. *EFSA Journal* 1-32 (<http://www.efsa.europa.eu/en/scdocs/doc/600.pdf>).
- Emna, S., Karim, A., Mohamed, K.C., Aida, B., 2006. Difficulty in dating primary infections by *Toxoplasma gondii* in pregnant women in Tunisia. *La Tunisie medicale* 84, 85-87.

- FAVV, 2008. Activiteitenverslag 2008, Federaal Agentschap voor de Veiligheid van de Voedselketen. 1-282.
- Fayer, R., 1981. Toxoplasmosis update and public health implications. *Can Vet J* 22, 344-352.
- Fehlhaber, K., Hintersdorf, P., Kruger, G., 2002. [Study on the prevalence of *Toxoplasma gondii* in pigs of different management systems and in minced meat]. *Fleischwirtschaft* 83, 97-99.
- Ferguson, D.J., 2004. Use of molecular and ultrastructural markers to evaluate stage conversion of *Toxoplasma gondii* in both the intermediate and definitive host. *Int J Parasitol* 34, 347-360.
- Ferguson, D.J., 2009. *Toxoplasma gondii*: 1908-2008, homage to Nicolle, Manceaux and Splendore. *Mem Inst Oswaldo Cruz* 104, 133-148.
- Ferguson, D.J., Birch-Andersen, A., Siim, J.C., Hutchison, W.M., 1979a. Ultrastructural studies on the sporulation of oocysts of *Toxoplasma gondii*. I. Development of the zygote and formation of the sporoblasts. *Acta pathologica et microbiologica Scandinavica. Section B, Microbiology* 87B, 171-181.
- Ferguson, D.J., Birch-Andersen, A., Siim, J.C., Hutchison, W.M., 1979b. Ultrastructural studies on the sporulation of oocysts of *Toxoplasma gondii*. II. Formation of the sporocyst and structure of the sporocyst wall. *Acta pathologica et microbiologica Scandinavica. Section B, Microbiology* 87B, 183-190.
- Ferguson, D.J., Birch-Andersen, A., Siim, J.C., Hutchison, W.M., 1979c. Ultrastructural studies on the sporulation of oocysts of *Toxoplasma gondii*. III. Formation of the sporozoites within the sporocysts. *Acta pathologica et microbiologica Scandinavica. Section B, Microbiology* 87, 253-260.
- Foulon, W., Naessens, A., Derde, M.P., 1994. Evaluation of the possibilities for preventing congenital toxoplasmosis. *Am J Perinatol* 11, 57-62.
- Frazaio-Teixeira, E., Sundar, N., Dubey, J.P., Grigg, M.E., de Oliveira, F.C., 2011. Multi-locus DNA sequencing of *Toxoplasma gondii* isolated from Brazilian pigs identifies genetically divergent strains. *Vet Parasitol* 175, 33-39.
- Fux, B., Rodrigues, C.V., Portela, R.W., Silva, N.M., Su, C., Sibley, D., Vitor, R.W., Gazzinelli, R.T., 2003. Role of cytokines and major histocompatibility complex restriction in mouse resistance to infection with a natural recombinant strain (type I-III) of *Toxoplasma gondii*. *Infect Immun* 71, 6392-6401.
- Gazzinelli, R.T., Denkers, E.Y., Sher, A., 1993. Host resistance to *Toxoplasma gondii*: model for studying the selective induction of cell-mediated immunity by intracellular parasites. *Infectious agents and disease* 2, 139-149.
- Grigg, M.E., Boothroyd, J.C., 2001. Rapid identification of virulent type I strains of the protozoan pathogen *Toxoplasma gondii* by PCR-restriction fragment length polymorphism analysis at the B1 gene. *J Clin Microbiol* 39, 398-400.

- Hay, J., Hutchison, W.M., 1983. *Toxoplasma gondii*--an environmental contaminant. *Ecol Dis* 2, 33-43.
- Hill, D.E., Haley, C., Wagner, B., Gamble, H.R., Dubey, J.P., 2010. Seroprevalence of and risk factors for *Toxoplasma gondii* in the US swine herd using sera collected during the National Animal Health Monitoring Survey (Swine 2006). *Zoonoses Public Health* 57, 53-59.
- Howe, D.K., Honore, S., Derouin, F., Sibley, L.D., 1997. Determination of genotypes of *Toxoplasma gondii* strains isolated from patients with toxoplasmosis. *J Clin Microbiol* 35, 1411-1414.
- Howe, D.K., Sibley, L.D., 1995. *Toxoplasma gondii* comprises three clonal lineages: correlation of parasite genotype with human disease. *J Infect Dis* 172, 1561-1566.
- Ji, Y.S., Sun, X.M., Liu, X.Y., Suo, X., 2013. *Toxoplasma gondii*: effects of exogenous nitric oxide on egress of tachyzoites from infected macrophages. *Exp Parasitol* 133, 70-74.
- Jones, J.L., Lopez, A., Wilson, M., Schulkin, J., Gibbs, R., 2001. Congenital toxoplasmosis: a review. *Obstetrical & gynecological survey* 56, 296-305.
- Kafsack, B.F., Pena, J.D., Coppens, I., Ravindran, S., Boothroyd, J.C., Carruthers, V.B., 2009. Rapid membrane disruption by a perforin-like protein facilitates parasite exit from host cells. *Science* 323, 530-533.
- Kijlstra, A., Eissen, O.A., Cornelissen, J., Munniksmma, K., Eijck, I., Kortbeek, T., 2004. *Toxoplasma gondii* infection in animal-friendly pig production systems. *Invest Ophthalmol Vis Sci* 45, 3165-3169.
- Kijlstra, A., Meerburg, B., Cornelissen, J., De Craeye, S., Vereijken, P., Jongert, E., 2008. The role of rodents and shrews in the transmission of *Toxoplasma gondii* to pigs. *Vet Parasitol* 156, 183-190.
- Kijlstra, A., Meerburg, B.G., Bos, A.P., 2009. Food safety in free-range and organic livestock systems: risk management and responsibility. *J Food Prot* 72, 2629-2637.
- Kijlstra, A., Petersen, E., 2014. Epidemiology, pathophysiology, and the future of ocular toxoplasmosis. *Ocular immunology and inflammation* 22, 138-147.
- Kim, J.H., Kang, K.I., Kang, W.C., Sohn, H.J., Jean, Y.H., Park, B.K., Kim, Y., Kim, D.Y., 2009. Porcine abortion outbreak associated with *Toxoplasma gondii* in Jeju Island, Korea. *Journal of veterinary science* 10, 147-151.
- Kravetz, J., 2013. Congenital toxoplasmosis. *Clinical evidence* 2013.
- Krishnan, L., Nguyen, T., McComb, S., 2013. From mice to women: the conundrum of immunity to infection during pregnancy. *J Reprod Immunol* 97, 62-73.
- Li, X., Wang, Y., Yu, F., Li, T., Zhang, D., 2010. An outbreak of lethal toxoplasmosis in pigs in the Gansu province of China. *J Vet Diagn Invest* 22, 442-444.

- Logar, J., Soba, B., Premru-Srsen, T., Novak-Antolic, Z., 2005. Seasonal variations in acute toxoplasmosis in pregnant women in Slovenia. *Clin Microbiol Infect* 11, 852-855.
- Meenken, C., Rothova, A., Kijlstra, A., Oosting, J., 1991. Seasonal variation in congenital toxoplasmosis. *Br J Ophthalmol* 75, 639.
- Meerburg, B.G., Van Riel, J.W., Cornelissen, J.B., Kijlstra, A., Mul, M.F., 2006. Cats and goat whey associated with *Toxoplasma gondii* infection in pigs. *Vector Borne Zoonotic Dis* 6, 266-274.
- Morrisette, N.S., Sibley, L.D., 2002. Cytoskeleton of apicomplexan parasites. *Microbiol Mol Biol Rev* 66, 21-38; table of contents.
- Nichols, B.A., Chiappino, M.L., O'Connor, G.R., 1983. Secretion from the rhoptries of *Toxoplasma gondii* during host-cell invasion. *Journal of ultrastructure research* 83, 85-98.
- Nicolle, C., Manceaux, L., 1908. Sur une infection á corps de Leishman (ou organismes voisins) du gondi. *CR Acad Sci*.
- Nicolle, C., Manceaux, L., 1909. Sur un protozoaire nouveau du gondi. *CR Acad Sci*.
- Nishi, M., Hu, K., Murray, J.M., Roos, D.S., 2008. Organellar dynamics during the cell cycle of *Toxoplasma gondii*. *J Cell Sci* 121, 1559-1568.
- Press, C., Montoya, J.G., Remington, J.S., 2005. Use of a single serum sample for diagnosis of acute toxoplasmosis in pregnant women and other adults. *J Clin Microbiol* 43, 3481-3483.
- Radke, J.R., White, M.W., 1998. A cell cycle model for the tachyzoite of *Toxoplasma gondii* using the Herpes simplex virus thymidine kinase. *Mol Biochem Parasitol* 94, 237-247.
- Robert-Gangneux, F., Darde, M.L., 2012. Epidemiology of and diagnostic strategies for toxoplasmosis. *Clin Microbiol Rev* 25, 264-296.
- Robert-Gangneux, F., 2014. It is not only the cat that did it: how to prevent and treat congenital toxoplasmosis. *J Infect* 68 Suppl 1, S125-133.
- Ryan, M., Hall, S.M., Barrett, N.J., Balfour, A.H., Holliman, R.E., Joynson, D.H., 1995. Toxoplasmosis in England and Wales 1981 to 1992. *Commun Dis Rep CDR Rev* 5, R13-21.
- Sagel, U., Mikolajczyk, R.T., Kramer, A., 2010. Seasonal trends in acute toxoplasmosis in pregnancy in the federal state of Upper Austria. *Clin Microbiol Infect* 16, 515-517.
- Schoeters, G., Hoogenboom, R., 2006. Contamination of free-range chicken eggs with dioxins and dioxin-like polychlorinated biphenyls. *Mol Nutr Food Res* 50, 908-914.
- Sibley, L.D., Boothroyd, J.C., 1992. Virulent strains of *Toxoplasma gondii* comprise a single clonal lineage. *Nature* 359, 82-85.
- Sibley, L.D., Khan, A., Ajioka, J.W., Rosenthal, B.M., 2009. Genetic diversity of *Toxoplasma gondii* in animals and humans. *Philos Trans R Soc Lond B Biol Sci* 364, 2749-2761.

- Sibley, L.D., Niesman, I.R., Parmley, S.F., Cesbron-Delauw, M.F., 1995. Regulated secretion of multi-lamellar vesicles leads to formation of a tubulo-vesicular network in host-cell vacuoles occupied by *Toxoplasma gondii*. *J Cell Sci* 108 ( Pt 4), 1669-1677.
- Splendore, A., 1908. Un nuovo protozoa parassita de' conigli. incontrato nelle lesioni anatomiche d'une malattia che ricorda in molti punti il Kala-azar dell' uomo. Nota preliminare pel. *Rev Soc Scient Sao Paulo*. 3, 109-112.
- Su, C., Zhang, X., Dubey, J.P., 2006. Genotyping of *Toxoplasma gondii* by multilocus PCR-RFLP markers: a high resolution and simple method for identification of parasites. *Int J Parasitol* 36, 841-848.
- Tenter, A.M., Heckeroth, A.R., Weiss, L.M., 2000. *Toxoplasma gondii*: from animals to humans. *Int J Parasitol* 30, 1217-1258.
- Thiptara, A., Kongkaew, W., Bilmad, U., Bhumibhamon, T., Anan, S., 2006. Toxoplasmosis in piglets. *Ann N Y Acad Sci* 1081, 336-338.
- Tizard, I.R., Fish, A., Quinn, J.P., 1976. Some observations on the epidemiology of toxoplasmosis in Canada. *J Hyg (Lond)* 77, 11-21.
- Tomavo, S., 2001. The differential expression of multiple isoenzyme forms during stage conversion of *Toxoplasma gondii*: an adaptive developmental strategy. *Int J Parasitol* 31, 1023-1031.
- Van Overmeire, I., Pussemier, L., Hanot, V., De Temmerman, L., Hoenig, M., Goeyens, L., 2006. Chemical contamination of free-range eggs from Belgium. *Food Addit Contam* 23, 1109-1122.
- Vanhaesebrouck, P., Foulon, W., Van Renterghem, L., Goossens, L., Temmerman, M., 2003. Perinatale *Toxoplasma gondii*-infectie, Een update anno 2002. *Tijdschr. voor Geneeskunde* 59, 663-671.
- Villari, S., Vesco, G., Petersen, E., Crispo, A., Buffolano, W., 2009. Risk factors for toxoplasmosis in pigs bred in Sicily, Southern Italy. *Vet Parasitol* 161, 1-8.
- Wong, S.Y., Remington, J.S., 1993. Biology of *Toxoplasma gondii*. *AIDS* 7, 299-316.

## **CHAPTER 2**

### ***TOXOPLASMA GONDII IN SHEEP***





In 1954 for the first time perinatal mortality due to toxoplasmosis was reported in sheep (Hartley, 1954). Meanwhile, it is known that the parasite infects sheep (*Ovis aries*) worldwide and is frequently being implicated in reproductive failure with severe economic losses as a consequence (Dubey, 2009; Dubey, 2010; Weissmann, 2003). In Europe, but also in most parts of the world, *T. gondii* is the second most important infectious cause of abortion in sheep and goats after *Chlamydia abortus* (Szeredi and Bacsadi, 2002). In some cases both pathogens can be detected during the same outbreak of abortion (Brodie et al., 1983; Steuber et al., 1995).

## **2.1 Transmission and pathogenesis**

### ***2.1.1 Oocysts in the environment***

Ovine Toxoplasmosis has been linked to feed or pasture contamination with sporulated oocysts. These oocysts may remain viable and infective in the environment for several months depending on temperature and relative humidity (Dubey, 1988; Innes et al., 2009b; Plant et al., 1974). Clinical toxoplasmosis starts by the ingestion of as few as 200 sporulated oocysts (McColgan et al., 1988). Associations have been made between infection of sheep and the ownership of cats and the presence of stray cats on the farms (McColgan et al., 1988). To prevent as much as possible contamination of sheep feed by cat faeces or infected rodents, sheep feed should be stored covered.

Once ingested, excystation of the oocysts takes place in the lumen of the small intestine, followed by the release of the sporozoites, which subsequently invade the intestinal mucosa, and multiply as tachyzoites in regional lymph nodes. This can occur already by the fourth day after ingestion. Then the endodyogeny takes place, during which *T. gondii* multiplies within the host cells, whereafter the parasitized cells rupture releasing the tachyzoites. These can then invade other host cells (Ferguson, 2009). In the meantime, the sheep develop fever that may persist until day 10 of infection. During this period, between six to eleven days after inoculation, the parasite may be found in the blood (parasitemia) during one or two days (Dubey and Sharma, 1980; Wastling et al., 1993). The haematogenous spread of the tachyzoites establishes a generalised infection. Meanwhile, tachyzoites transform into bradyzoites under influence of the host immune response. Bradyzoites, sequestered in tissue cysts, maintain the infection and a lifelong immunity.

### ***2.1.2 Congenital Transmission***

Besides oral infection with oocysts, the only other route of infection is vertical from mother to foetus during pregnancy. This represents a serious risk for congenital disease (Dubey, 2009). Disease signs are depending on the immune status of the ewe and the stage of gestation at the time of infection (Brodie et al., 1983). They vary from foetal resorption, mummification, abortion, stillbirth, to birth of weak lambs or lambs with growth retardation (Buxton, 1998). Abortions due to toxoplasmosis are particularly seen in temperate countries, where climatic conditions favour oocyst survival and sporulation (Innes et al., 2007). The naive and pregnant ewe is susceptible to *Toxoplasma*-induced reproductive losses when parasitaemia occurs and tachyzoites invade the uterus and placenta. In case the primary infection takes place before the last month of gestation; the consequences for the foetus are more severe like abortion, foetal death and mummification due to the absence of a foetal immune response (Blewett et al., 1982; Hartley and Moyle, 1974). There is no inhibition of parasite multiplication what causes foetal resorption and the ewes subsequently will be barren.

A primary infection in the later stages of pregnancy (after day 120 of gestation) will result most of the time in the birth of a normal lamb, which may be infected and immune. At that stage, the immune system of the foetus is already sufficiently developed (Blewett and Watson, 1983; Buxton et al., 2006; Rodger et al., 2006). The interval between infection and abortion is estimated to be about forty days, similar to enzootic abortion due to *Chlamydia abortus* (Bos, 1993). Approximately five days after *T. gondii* infection, pregnant ewes develop pyrexia that lasts for four days (Owen et al., 1998). During this period, the tachyzoites spread to the placenta where they invade and multiply within the maternal caruncular septa in the placentome and from there they invade the adjoining foetal trophoblast cells (Buxton and Finlayson, 1986). Placental tissue from infected ewes may show characteristic gross white spot lesions, areas of necrosis that will limit its function in supporting the pregnancy (Fredriksson et al., 1990). Placentitis occurs, and abortion as well as stillbirth may be attributed to placental insufficiency (Owen et al., 1998). Pyrexia may also induce abortion in the acute phase of the infection (Owen et al., 1998). The immune balance between the ewe and the foetus, plays another important role in the severity of the disease (Anderson et al., 1994; Innes and Vermeulen, 2006). In subsequent pregnancies the immune response developed by the infected animals, will protect them against disease (Innes et al., 2009b). A non-pregnant ewe, infected with *T. gondii*, will equally develop an immune response that protects against *Toxoplasma*-induced abortion during a following gestation (Buxton, 1998;

Rodger et al., 2006). However, data of Morley et al. (2005, 2008) suggest that immunity might be less protective against transplacental transmission than thought. In a first paper, abortion data of 765 ewes from 27 Charollais sheep families in one flock were investigated. The abortion rate for the families varied from 0 up to 100 %. Tissue samples of 155 aborted lambs were analysed for *T. gondii* DNA. The positivity varied also from 0-100 % (Morley et al., 2005). In a follow up study 29 ewes from the first report that gave birth to lambs during two successive years, were selected. Nine of the ewes produced 35 lambs over two lambings. From those 35, 37 % were born alive and 63 % were aborted or mummified. Thirty-three out of those 35 lambs were PCR-positive when tested with SAG1 nested PCR (Morley et al., 2008). However, those studies are based only on *T. gondii* DNA detection. Hence there is neither demonstration of *T. gondii*-associated lesions by histopathology nor exclusion of other abortifacient agents (Dubey, 2009).

### ***2.1.3 Recrudescence of an endogenous infection***

Recrudescence of an endogenous infection is a common transmission route of congenital infection with *Neospora caninum* in cattle (Innes et al., 2005). Endogenous transplacental transmission of *T. gondii* occurs very infrequent and is not considered as a clinical risk (Dubey, 1988; Innes et al., 2009b). However, the endogenous transplacental transmission of *Toxoplasma* was suggested to play a more significant role than previously thought (Duncanson et al., 2001; Williams et al., 2005). All in all, the studies conclude that endogenous transplacental transmission could occur in persistently infected ewes, but that is very rare. Other studies have shown that the seroprevalence increases with age meaning that most infections occur after birth and suggest that the most significant cause of infection for sheep is *T. gondii* oocysts in the environment and not by vertical transmission (Blewett and Watson, 1983; Waldeland, 1977).

## 2.2 Immunological Responses

Host protection against *T. gondii* is complex and takes into account innate and adaptive immune mechanisms and within the latter both humoral as well as cellular immunity play a role. During the acute infection high levels of *T. gondii* specific serum antibodies can appear (Dubey, 1988). After feeding oocysts to sheep, latex agglutination test (LAT) titers increased eightfold and these antibodies could be detected earlier than by an indirect immunofluorescence antibody test (IFA) or by an indirect hemagglutination assay (IHA) (Trees et al., 1989). It is well known that agglutination tests detect IgM antibodies easier than IgG. IgM antibodies appear 10-12 days after a primo-infection and peak at three weeks post infection. They precede the IgG response (Innes et al., 2009b; Trees et al., 1989). Once IgG is produced, it remains in the blood circulation lifelong. High IgG antibody levels can persist in sheep for months or years (Dubey, 1988). Re-infection does not increase the antibody titer (McColgan et al., 1988)

The cell-mediated immunity is seen as the mediator for protection. In several studies in mice the role of cytokines, antibodies and immune cells, especially CD4<sup>+</sup> and CD8<sup>+</sup> T cells has been investigated (Denkers and Gazzinelli, 1998; Frenkel, 1967; Gazzinelli et al., 1991; Suzuki et al., 1988; Yap and Sher, 1999). Mainly interferon-gamma (IFN-gamma) seems the pre-eminent cytokine for protection (Ely et al., 1999; Gazzinelli et al., 1993a; Gazzinelli et al., 1993b; Khan et al., 1994; Suzuki et al., 1988). CD4<sup>+</sup> as well as CD8<sup>+</sup> T cells are the main producers of IFN-gamma, but CD8<sup>+</sup> T cells play a more crucial role in protective immunity *in vivo* (Gazzinelli et al., 1991; Suzuki et al., 1989). Depletion of IFN-gamma in chronically infected mice led to reactivation of *T. gondii* tissue cysts (Suzuki et al., 1989).

As mentioned above, most of the research has been done in mouse models. There are only a few studies done in sheep. Three days after experimental *T. gondii* infection of sheep, parasites were detected in the efferent lymph, IFN-gamma comes up one day later, preceding the detection of lymphoblasts (Innes et al., 1995a; Innes et al., 1995b). Subsequently, there is a switch from CD4<sup>+</sup> T cells, initially the largest population, to CD8<sup>+</sup> cells (Innes et al., 1995a). This switch is accompanied with the disappearance of parasites from the lymph. Other evidence demonstrating the importance of IFN-gamma, is that ovine recombinant IFN-gamma inhibits *Toxoplasma* replication in ovine fibroblasts and macrophages (Oura et al., 1993). Activated CD8<sup>+</sup> T cells are also able to inhibit *T. gondii* multiplication within autologous target cells (Innes et al., 1995a). In general, CD4<sup>+</sup> as well as CD8<sup>+</sup> T cells are necessary for the development of protective immunity to *T. gondii*.

## 2.3. Diagnostic detection methods

Many detection techniques are available for the diagnosis of *T. gondii*, however with different sensitivities and specificities. Therefore, in some cases a combination of different techniques is preferable.

### 2.3.1 Serological methods

There is still no serological test validated for the detection of *T. gondii* in sheep when compared to the gold standard "isolation of the parasite" (Dubey, 2009). Nonetheless, serological analysis using tests such as indirect immunofluorescent antibody test (IFA), an enzyme-linked immunosorbent assay (ELISA) and/or a modified agglutination test (MAT) has been widely and commonly employed in order to demonstrate infection of *T. gondii* in sheep flocks in several countries (van der Puije, 2000). Both IgG and IgM are demonstrated for diagnosing ovine toxoplasmosis.

#### 2.3.1.1 Sabin-Feldman dye test

The Sabin Feldman test (SFDT), also known as *T. gondii* lysis test or dye test, was first described sixty years ago by Sabin and Feldman (Sabin and Feldman, 1948). A first step towards the standardization was taken in 1968 when the WHO recommended the expression of dye test titres in international units per millilitre. A first international human standard serum for total anti-*Toxoplasma* antibodies was then produced, followed by a second one in 1980. The estimation of IU in both preparations is based on dye test results for the detection of all immunoglobuline classes using membrane antigens on the surface of live *T. gondii* (Reiter-Owona et al., 1999; Standardization, 1968, 1981). The SFDT – still regarded as the gold standard - and thus the reference test for human toxoplasmosis. It has the highest sensitivity and specificity (Reiter-Owona et al., 1999), thus detect early and late *Toxoplasma* infections.

The SFDT is based on complement-mediated cytolysis of antibody-coated live *T. gondii* tachyzoites and uptake of methylene blue dye by living tachyzoites. Seronegative human serum used as an accessory complement factor and live *T. gondii* tachyzoites are added to samples of diluted patients' serum. After an incubation for 1 hour at 37°C, methylene blue is added and each reaction is read using a polarised light microscope. If *Toxoplasma* tachyzoites are not stained, cytolysis due to the presence of anti-*Toxoplasma* antibodies in the patients'

serum has occurred and the test is positive. If tachyzoites appear blue under the microscope, they have an intact membrane and therefore no specific antibodies were present in the serum. Nowadays the use of methylene blue is used as a confirmation. The test is mostly read by phase contrast microscopy, allowing the observation of cells and organelles on unstained, living parasites (McGovern and Wilson, 2013). Although this test is the most specific test for *T. gondii*, it has several disadvantages such as: the necessity of live virulent tachyzoites, the requirement of human serum from seronegative individuals as complement, the impossibility to automate the test, the time needed to read each dilution and thus the high cost. This technique has therefore been replaced by other tests in most laboratories (Dubey, 1988; Shaapan et al., 2008).

#### ***2.3.1.2 Indirect immunofluorescent antibody test (IFA)***

The indirect immunofluorescent antibody test (or assay; IFA) uses intact formalin-fixed tachyzoites. The tachyzoites are coated on a microscope slide and incubated with dilutions of the serum to be tested. If specific antibodies are present, they will bind to the parasite. The slides are then incubated with, a fluorescent molecule labelled, secondary antibody which is species and immunoglobulin specific. Fluorescein isothiocyanate (FITC) is commonly used. Evans blue is added to the FITC labelled conjugate for counterstaining (Ambroise-Thomas et al., 1966).

After washing and drying, the slides are examined by fluorescence microscopy. A positive reaction colours green, a negative reaction will be red when counterstained with Evans Blue.

The limitation of this technique, as with most fluorescence techniques, is the loss of fluorescent activity and thus resolution caused by photo bleaching. The major disadvantage of this test is the complexity of reading and therefore the need for well-trained personnel. The subjectivity of the visual assessment of fluorescence of the whole-cell antigen can be controlled by reducing light exposure or by employing more robust fluorophores such as Alexa fluors (Ambroise-Thomas et al., 1980; Gordon et al., 1981).

This method is highly specific, but a lower sensitivity in sheep has been demonstrated (Shaapan et al., 2008). Nevertheless, the IFA is still more sensitive and specific than the other widely used methods such as IHA, LA and ELISA. The technique is reproducible, with antibody titers related to clinical events (Gordon et al., 1981).

### **2.3.1.3 Enzyme-Linked Immunosorbent Assay (ELISA)**

The enzyme-linked immunosorbent assay is used to detect specific antibodies against *T. gondii* in sera. Crude, fractionated and recombinant antigens are widely used in various ELISA methods.

Anti-*Toxoplasma* IgM antibodies are considered as a main marker of acute toxoplasmosis (Naot and Remington, 1980). However, when testing for IgM, major difficulties are encountered such as the persistence of positive values (Fuccillo et al., 1987), the lack of specificity associated with immunopathological responses (Fuccillo et al., 1987; Konishi, 1987; Liesenfeld et al., 1997; Palosuo and Aho, 1983). As a consequence, IgM is often not a good marker to have an idea regarding the time of the infection and additional methods for determining acute toxoplasmosis, such as IgG avidity assays are necessary. Avidity is the affinity with which IgG attaches to the antigen and this matches with the time following a primary infection. In other words, IgG produced within the first months following primary infection exhibits low avidity, whereas IgG produced months or years later exhibits high avidity levels (Hedman et al., 1989; Joynson et al., 1990). Studies of the kinetics of the IgG avidity in sheep, showed that sheep with high avidity IgG were infected at least 10 weeks earlier (time to conversion from low to high avidity antibodies varies with the method used) (Sager et al., 2003b). Similar results are found in humans (Auer et al., 2000). Most of the avidity tests use urea, or other denaturing agents that disrupt van der Waals' interactions. Low avidity IgG readily dissociates from the antigen in the presence of urea whereas high avidity IgG does not (Rossi, 1998). Especially in case of a possible human congenital infection, avidity testing may be important for identifying pregnancies that are at risk for congenital toxoplasmosis (Clementino et al., 2007; Sager et al., 2003a; Sager et al., 2003b). However, a study of Jenum et al. (1997) showed that, in some cases, a low avidity could persist for more than 20 weeks. So in these cases a low IgG avidity could not identify a recent acquired infection in the first 13 to 20 weeks of the pregnancy (Auer et al., 2000; Hedman et al., 1989; Jenum et al., 1997). Because ELISA is quantitative, highly sensitive and has a low cost it is a commonly used test for diagnosing infection in sheep. Nonetheless, the lack of standardization of this technique and of the coated antigens is a major problem (Dubey et al., 1995).

### **2.3.1.4 Agglutination**

A direct agglutination test (DT) was first described 50 years ago by Fulton and Turk (1959). The original method entails the reaction of specific antibodies, present in the sample to be tested, with whole formalin-fixed *Toxoplasma* tachyzoites. Important disadvantages of this test are the lack of sensitivity and specificity. In general, the titer in the agglutination test is in general higher than in the Sabin-Feldman dye test (SFDT) or the IFA. Some sheep sera are seen as positive in agglutination while they are negative in SFDT and IFA (Shaapan et al., 2008). The reason is the binding of IgM to the surface of the parasite (Desmonts and Remington, 1980). Subsequent modifications were developed to increase the sensitivity and specificity. One method is to use 2-mercaptoethanol in the incubation buffer. This will reduce the disulphide bridges in the IgM, making it fall apart into its monomers. Destroying the IgM decreases non-specific reactions (Dubey et al., 1987). Other alternatives for the DT are the latex agglutination test (LAT) and the indirect hemagglutination test (IHA) (Lunde and Jacobs, 1959). In the IHA, sheep red blood cells were used as antigen carrier and in the LAT latex particles (Balfour et al., 1982; Jacobs and Lunde, 1957). An agglutination test is simple to perform, but due to the use of 2-mercaptoethanol in the incubation buffer, only IgG antibodies can be demonstrated, making this method not useful for diagnosing early infections.

### **2.3.2 Detection of *T. gondii***

Direct detection of the parasite is conducted in most of the cases to confirm dubious or uncertain serological results. However, in veterinary diagnosis, those tests are mainly used to confirm infection in aborted foeti or dead animals.

#### **2.3.2.1 Bio-assay**

##### **2.3.2.1.1 Bio-assay in mice**

Clinical samples are intraperitoneally inoculated into female *T. gondii*-free Swiss mice. Blood samples are taken from these mice after an observation period of 45 days and individually examined for the presence of anti-*Toxoplasma*-specific IgG antibodies by IFA. Mice having received positive and thus infectious sample will seroconvert, while those inoculated with negative material will remain negative. A confirmation of the parasite infection is then performed on the brains by PCR or by light microscopy to demonstrate the



presence of cysts. Due to the low concentration of the cysts is the use of microscopy often difficult. Three smears have to be examined, at least in case of low mouse virulent parasite strains (Jacobs and Melton, 1954). Indeed, during acute infection tachyzoites can be found in peritoneal exudate of mice, but tachyzoites may be rare when infected with a low virulent strain (Derouin et al., 1987).

The susceptibility of mice to *T. gondii* depends on the infection route, the challenge dose (Eyles and Coleman, 1956) and the parasite stage (Derouin et al., 1987). Chronic infection is seen after inoculation with bradyzoites of a low virulent strain. Inoculation with tachyzoites of type I strains causes death. However, the strain is equally important (Johnson, 1984). Some strains infect mice poorly, others strongly (Jacobs and Melton, 1954). Tachyzoites of the RH strain kill mice from acute infection within 9 days.

Bioassay is still regarded as the reference method to detect viable *T. gondii* in tissues or fluids, Many authors consider it as the most sensitive method even though this method has the disadvantages of being labour-intensive, using animals and it can take 30 to 45 days incubation to obtain the result (Derouin et al., 1987; Hitt and Filice, 1992). The sensitivity is 1 cyst per 100 gram tissue (Rothe et al., 1985). However, more rapid and highly reliable diagnostic methods have to be used in clinical cases where early therapeutic treatment is necessary.

#### 2.3.2.1.2 Bio-assay in cats

The number of tissue cysts in sheep or other food producing animals is sometimes too small to be detectable by the bioassay in mice. Larger volumes of minced sheep tissue can be fed to cats. The administration of 500 g meat or more (per cat) makes this technique more sensitive than the bioassay in mice (Dubey, 2010). The major drawback of this test is the shedding of very resistant oocysts by the inoculated cats, which can lead to environmental contamination and are a serious risk for laboratory personnel; a lot of safety measures have to be taken into account using this test. Purification and concentration of oocysts from faeces is done by centrifugation (Kourenti et al., 2003), flocculation (Dubey et al., 1970; Kourenti et al., 2003) and flotation techniques (Dubey et al., 1970). Hereto, faeces are first mixed with distilled water and poured through a strainer, followed by centrifugation. After several washing steps, the oocysts eventually present in the pellet can be isolated by a standard flotation technique using a sucrose (Dubey et al., 1970) or cesium chloride solution (Dumetre and Darde, 2004). Detection of the purified oocysts can be achieved by light or fluorescence microscopy, bioassay in mice or PCR (coproPCR).

Confusion between *T. gondii* oocysts and those from related coccidians hampers the detection by light microscopy as well as the insufficient concentrating of the oocysts. Unsporulated oocysts are 10 x 12 µm, smaller than *Isospora felis* oocysts and *Toxocara cati* eggs, but bigger than red blood cells (Dubey, 2010). Extra attention has to be paid to distinguish the *T. gondii* oocysts from those from *Hammondia hammondi*. The only differentiating method between *T. gondii* and *Hammondia sp.* is by PCR or by mouse inoculation after sporulation of those oocysts (Dubey, 2010).

Fluorescence microscopy is based on a blue autofluorescence of the oocysts (Lindquist et al., 2003). The intensity and the time of being auto fluorescent are sufficient enough to see oocysts. However, due to the autofluorescence of related coccidia, additional methods are needed to clearly identify the *T. gondii* oocysts (Lindquist et al., 2003). Further information is given in 2.6.2.4.3.

PCR is very sensitive but before the DNA extraction can be performed, the oocysts have to be purified and concentrated from the fecal matter. Furthermore, the presence of some substances like clay, blood and polysaccharides can inhibit the detection.

### **2.3.2.2 Cell Culture**

This technique provides also information about the parasite's growth, replication and structure with no interference of immune effector mechanisms (Azab and Beverley, 1974; Hughes et al., 1986). *In vitro* easily growing cell lines can be used to culture *T. gondii*, but usually fibroblast cell lines such as the human embryonic fibroblast cell line, MRC5, are used. *Toxoplasma* can already be detected in the cells using an immunofluorescence technique (described in 2.6.2.4.3) from 2 days after inoculation. However, later examination of the cell cultures, on days 4 to 5 after inoculation, is preferable for easier identification of the parasite. Indeed, clusters of *T. gondii* can be observed after four days incubation (Hitt and Filice, 1992; Hughes et al., 1986).

Although much less sensitive than bioassay, the cell culture was the most practical and readily available technique for *T. gondii* detection, until PCR was developed. Nowadays this method is abandoned by the laboratories (Hitt and Filice, 1992).

### **2.3.2.3 Polymerase Chain Reaction (PCR)**

Molecular detection methods such as PCR are increasingly used in veterinary and human medicine to diagnose toxoplasmosis (Fuentes et al., 1996; Montoya et al., 2009). After all, isolation of *T. gondii* DNA from placental and foeti tissues confirms the diagnosis and is very useful in recovering the parasite from autolysed tissues as those may be useless for histological examination or bioassay (Dubey, 2010). Many PCR based assays have been developed for the detection of *T. gondii* DNA in blood, fluids and tissue samples. The most common PCR *T. gondii* target is the B1 repetitive sequence, which is a 35-copy repeated DNA sequence (Burg et al., 1989; Burg et al., 1988). This protocol was developed in 1989 and has since then been modified and optimized by many laboratories (Fuentes et al., 1996; Liesenfeld et al., 1994). Other targets are the *p30* or *Sag1* gene, with only one copy and a more and more used 529 bp fragment that is repeated 200- to 300-fold in the *T. gondii* genome (Chabbert et al., 2004; Ellis, 1998; Homan et al., 2000).

PCR can be applied on various types of samples and is specific, sensitive as well as fast in comparison with the other methods (MacPherson and Gajadhar, 1993; van de Ven et al., 1991). Even if the samples are in a decomposed state, detection of DNA from the parasites is possible whilst a bioassay only detects viable parasites (Yai et al., 2003). Despite these advantages, diagnosis by PCR remains limited due to the still expensive equipment (Kong et al., 2012). Additionally, false positive results have been reported due to cross-reactivity e.g. with species of *Nocardia*, *Mycobacterium* and *Actinomyces* (Bhalla et al., 1999). Furthermore, false negatives can occur due to the presence of inhibitory factors e.g. porphyrin (Dupon et al., 1995; van de Ven et al., 1991).

A modification of the polymerase chain reaction is the nested-PCR. This method is used to increase the sensitivity and specificity of amplification (Fallahi et al., 2014). The technique uses two pairs of primers, the external ones and the internal ones. The external primer pair has a higher melting temperature and is used in lower concentration than the internal ones, to prevent the first amplification round interfering with the second one (Ellis, 1998). The PCR product generated with the external primer pair, is used as a template for the second PCR. The binding sites of the second primer set are nested within the first set, which enhances the specificity (Pujol-Rique et al., 1999). Even though PCR is often used for diagnosing the cause of an abortion and as screening method in flocks, unfortunately until now no standardised method or commercial kit is available; different sets of primers and different PCR products are used by different laboratories.

Competitive PCR is a commonly used method to transform PCR from a qualitative method into a quantitative assay (Orlando et al., 1998). The technique includes a known quantity of an exogenous competitor molecule (also known as a PCR mimic) which competes with the target for the components of the PCR since it is amplified by the same primers as the target sequence. The known quantity of PCR mimics are spiked into the PCR amplification reactions containing the target DNA. During the reaction, the amount of products amplified relates directly to the amount of template molecules (both genomic and competitor targets) in the original reaction. Quantitation is then performed by comparing the amount of target PCR product with the amount of product amplified from a defined quantity of competitor (Orlando et al., 1998; Siebert and Larrick, 1993). Competitive PCR was initially followed by agarose gel image analysis for quantification of the parasite load in the sample, which was a time consuming procedure (Homan et al., 2000; Kirisits et al., 2000; Luo et al., 1997).

Nowadays real-time PCR is commonly used for quantification of the parasitic load (Jauregui et al., 2001). This technique follows the general principle of a PCR with the extra ability to monitor the progress of the PCR as it occurs, i.e. in “real-time”. The reactions are characterized by the time-point when the amplification of a target is first detected, rather than the amount of accumulated product after the number of cycles. The higher the starting quantity of the DNA target, the sooner a significant increase in fluorescence is observed. In contrast, the amplified DNA in a standard PCR is detected at the end of the reaction (Fraga et al., 2014). The sensitivity of real-time PCR can be greatly enhanced using a magnetic capture technique: a crude DNA extract is prepared from a 100 g meat sample and subjected to a *T. gondii* DNA concentration step using magnetic beads coated with probes that target a specific region on the *T. gondii* genome. After this capture step, real-time PCR can be performed (Opsteegh et al., 2010a).

### **2.3.2.4 Microscopy**

#### **2.3.2.4.1 Light microscopy**

Light microscopy, and later on fluorescence and electron microscopy have been intensely used for studying the parasite since Nicolle and Manceau described *T. gondii* in 1908. Jankû has published the first images of *T. gondii* in 1923. The photograph was taken from the retina of an infant. The tachyzoites were described by Jankû (1923) as “small, cylindrical bat-like cells in rosette form”. Tachyzoites were first observed by Splendore as structures with “yellow cytoplasm and granular nucleus” (Splendore, 1908), he noted that they are better

visualised after Giemsa staining, by colouring the negatively charged phosphate groups on DNA. Hematoxylin and eosin (H&E) staining is also frequently used for visualising tachyzoites. Hematoxylin stains nuclear proteins, violet coloured complexes are formed with metal ions, while eosin, a red acidic dye, colours the proteins (Jankû, 1923). A periodic acid Schiff (PAS) staining visualises the tachyzoites poorly. The bradyzoites contain several amylopectin granules which stain red with PAS reagent. Those granules are in discrete particles or even absent in tachyzoites. The periodic acid oxidizes the polysaccharides whereafter they react with the Schiff component resulting in a pink-purple colour (Dubey et al., 1998).

*T. gondii* tissue cysts are strongly stained with H&E. Also Giemsa, Mallory and Biondi staining of cysts result in clear images. Mallory is a mix of acid fuchsin, aniline blue and orange G, three dyes which the nuclei red, cytoplasm pink and the extracellular matrix blue. Biondi consists of aurantia, acid fuchsin and methyl-green which colours the chromatin green and cytoplasm pale yellow (Jankû, 1923).

With a Silver and PAS colouring it is easier to visualise the cysts. However, confusion with cysts of other parasites is possible (Sims et al., 1988). Therefore and due to the limited resolution, light microscopy is an inferior method for identifying tissue cysts (McGovern and Wilson, 2013). But, it allows to determine the complete cycle of *T. gondii* (Dubey and Frenkel, 1972).

Histologic and cytologic examinations are still used to diagnose *T. gondii*-induced abortion in ewes. *T. gondii* causes no macroscopic lesions in the foetus but produces microscopic to macroscopic necrosis of the placental cotyledons. Recognizing lesions is more important than to find the parasite, because degenerating host cells are often confused with *T. gondii* tachyzoites (Dubey, 2010).

#### 2.3.2.4.2 Fluorescence and bioluminescence

As mentioned above, oocysts exhibit a blue autofluorescence, which makes them visible under UV-light (Lindquist et al., 2003). In addition, most proteins of interest can be visualised by staining them with fluorescently labelled protein-specific antibodies (Nery-Guimaraes et al., 1968).

#### 2.3.2.4.3 Confocal microscopy

The basis of the confocal microscopy was patented in 1957 by Marvin Minsky of the Harvard University. The first confocal microscope dates from 1979. The technique of laser scanning confocal fluorescence microscopy is especially used to study the subcellular structures of *T. gondii* (Melzer et al., 2010). The main advantages in comparison with the conventional optical microscope are the inclusion of shallow depth of field, the elimination of out-of-focus glare and the ability to make a series of optical sections from thick specimens. In the case of *T. gondii* the microscope is used to make ultra-structural images of fixed or living parasites that have been labelled with one or more fluorescent probes. The main disadvantage of this type of microscopy is the high cost of the equipment (Kervrann et al., 2000).

## **2.4 Seroprevalence**

The seroprevalence of *T. gondii* in sheep has been investigated worldwide and found to vary greatly (Dubey, 2009). Caballero-Ortega *et al.* (2008) suggested a relation between seropositivity and altitude, being higher at sea level than at 1200 meters above sea. Furthermore the farm size was important and seropositivity was higher in larger than in small flocks (Caballero-Ortega et al., 2008). Table 1 presents seroprevalence data per country from 1991-2013.

**Table 1:** Seroprevalence data from 1991-2013 of *T. gondii* per country and the serological assays used.

Country	# animals	test	Commercial/method	Cut-off titer	% Pos	Reference
Argentina	704	IFA	ns	50	17,3	Hecker et al. (2013)
Austria	4079	IFA	ns	40	66,4	Edelhofer and Aspöck (1996)
Bangladesh	25	LAT	Toxotest-MT® (Eiken Kagaku, Tokyo, Japan)	8	40	Shahiduzzaman et al. (2011)
Brazil	382	MAT	In house test according to the method of Desmonts and Remington (1980)	16	18,6	Langoni et al. (2011)
	360	IFA	In house test according to the method of Camargo (1964)	64	56,9	Sakata et al. (2012)
	795	IFA	In house test	64	30,2	Guimaraes et al. (2013)
	932	IFA	In house test	64	28,22	Mendonca et al. (2013)
	930	ELISA	In house test according to the method of Clementino et al., (2007)	ns	22,1	Andrade et al. (2013)
Bulgaria	380	IHA	In house test	1280	48,2	Prelezov (2008)
Burkina Faso	65	IHA	ns	64	23	Deconinck (1996)
Canada	3872	ELISA	TLA RH (IgG)	ns	57,6	Waltner-Toews et al. (1991)
China	580	IHA	Kit Lanzhou® ( Vet. Res. Inst. Chinese academy of agriculture)	64	4,4	Liu et al. (2010)
	455	IHA	Kit Lanzhou® (Vet. Res. Inst. Chinese academy of agriculture)	64	5,7	Wu et al. (2011)
	566	IHA	Kit Lanzhou® (Vet. Res. Inst. Chinese academy of agriculture)	64	29,8	Yang et al. (2013)
Czech Republic	24	IFA	ns	40	4	Sedlák and Bártová (2007)
	547	ELISA	Commercial kit (Institut Pourquier, Montpellier, France)	ns	59	Bartova et al. (2009)
Cjile	408	IFA	In house test	16	28	Gorman et al. (1999)
Côte d'Ivoire	62	IHA	ns	64	68	Deconinck (1996)
Djibouti	183	IHA	ns	64	13	Deconinck (1996)

Egypt	300	MAT	In house test according to method of Dubey and Desmonts (1987)	25	43,7	Shaapan (2008)
	320	IHA	ns	80	47,5	Barakat et al. (2009)
	62	ELISA	In house test according to the method of Voller et al. (1976)	ns	98,4	Ghoneim et al. (2010)
Ethiopia	116	MAT	Toxosreen DA test (®Behring, Germany)	ns	52,6	Negash et al. (2004)
	787	P-30 ELISA	Kit ID Screen® (ID. Vet Montpellier France)	ns	37	Gebremedhin et al. (2013b)
	357	P-30 ELISA	Kit ID Screen® (ID. Vet Montpellier France)	ns	31,59	Gebremedhin et al. (2013a)
France	141 lambs	MAT	In house test according to method of Desmonts and Remington (1980)	20	20,5	Rozette (2005)
	65 ewes				59,1	
	164 lambs	MAT	In house test according to method of Desmonts and Remington (1980)	20	22	Dumetre et al. (2006)
	93 ewes				65,6	
Finland	1940	MAT	Toxosreen DA test® (BioMérieux, Marcy-l'Etoile, France)	40	24,6	Jokelainen et al. (2010)
Germany	1122	ELISA	ns	ns	33	Seineke (1996)
Great Britain	411	MAT	In house test according to method of Dubey and Desmonts (1987)	25	12,2	Mason et al. (2010)
	329				3	
	3539	LAT	accredited test used by Animal Health and Veterinary Laboratories Agency UK	64	74	Hutchinson et al. (2011)
Greece	1501	tg SAG1- ELISA	In house test	ns	48,6	Tzanidakis et al. (2012)
	458	ELISA	In house test according to the method of Osawa et al. (1998)	ns	53,71	Anastasia et al. (2013)
	360	TLA- ELISA	In house test	ns	28,3	Kantzoura et al. (2013)
Ghana	732	TLA- ELISA	In house test according to the method of Voller et al. (1976)	ns	33	van der Puije (2000)
India	186	ELISA	Commercial kit (Institut Pourquier, Montpellier, France)	ns	3,8	Sharma (2008)
	204	MAT	In house test according to method of Dubey and Desmonts (1987)	25	44,1	Chikweto et al. (2011)



Iran	588	IFA	In house test according to method of Voller and O'Neill (1971)	16	35	Sharif et al. (2007)
	105	LAT	Commercial kit (®LA, SGM Italia, Roma Italy) according to method of Mazumder et al. (1988)	8	20,9	Zia-Ali et al. (2007)
	150	ELISA	Commercial kit (Institut Pourquier, Montpellier, France)	ns	72,6	Hamidinejat et al. (2008)
	156	MAT	In house test according to method of Desmonts and Remington (1980)	20	21,1	Raeghi et al. (2011)
Ireland	292	LAT	Toxoplasma LAT test® (Toxoreagent RST701, Mastgroup Ltd., Merseyside, UK)	64	36	Halova et al. (2013)
Italy	7194	IFA	Toxo-spot IF® (BioMérieux Marcy-l'Etoile, France)	200	28,4	Masala et al. (2003)
	1056	LAT	Toxotest-MT® (Eiken Kagaku, Tokyo, Japan)	32	78	Gaffuri et al. (2006)
	1876	ELISA	Commercial kit (Institut Pourquier, Montpellier, France)	ns	49,9	Vesco (2007)
	1170	IFA	Toxo-spot IF® (BioMérieux Marcy-l'Etoile, France)	100	28,5	Fusco et al. (2007)
	630	IFA	IFA slides® (Mega-Cor diagnostic, Austria)	64	34	Cenci-Goga et al. (2013)
Japan	267	IFA	Toxo-spot IF® (Biomérieux, Marcy-étoile, France)	40	28,78	Giangaspero et al. (2013)
Lithuania	354	ELISA	Chekit Toxotest Enzyme Immunoassay® (IDDEXX laboratories, Intervet, The Netherlands)	ns	42,1	Stimbirys et al. (2007)
Mexico	511	MAT	In house test according to the method of Dubey and Desmonts (1987)	25	15,1	Alvarado-Esquivel et al. (2012)
	429	MAT	In house test according to the method of Dubey and Desmonts (1987)	25	23,1	Alvarado-Esquivel et al. (2013a)
	405	MAT	In house test according to the method of Dubey and Desmonts (1987)	25	29,9	Alvarado-Esquivel et al. (2013b)
Mongolia	175	LAT	Toxotest-MT® (Eiken Kagaku, Tokyo, Japan)	32	16,57	Tumurjav et al. (2010)
Netherlands	1179	ELISA	In house test	ns	30	Opsteegh et al. (2010b)
New Zealand	2254	LAT	Toxotest-MT® (Eiken Kagaku, Tokyo, Japan)	16	85	Dempster et al. (2011)
Niger	77	IHA	ns	64	19,5	Deconinck (1996)
Nigeria	372	ELISA	Commercial kit (Institut Pourquier, Montpellier, France)	ns	6,7	Kamani et al. (2010)
Norway	1940	ELISA	In house test	ns	16,2	Skjerve et al. (1998)
Pakistan	90	LAT	Toxotest-MT® (Eiken Kagaku, Tokyo, Japan)	8	11,2	Ramzan et al. (2009)

Peru	211	IHA	Commercial antigen (TPM-TEST Wampole laboratories, Cranbury, NJ)	64	27	Reif et al. (1989)
Poland	41	IFA	ns	8	53,6	Gorecki et al. (2008)
Portugal	119	MAT	Toxosreen DA test® (BioMérieux, Marcy-l'Etoile, France)	20	33,6	Lopes et al. (2013)
Scotland	3333	ELISA	In house test according to the method of Buxton et al. (1988)	25%	56,6	Katzer et al. (2011)
Senegal	52	IHA	ns	64	11,5	Deconinck (1996)
Serbia	511	MAT	In house test according to the method of Desmonts and Remington (1980)	25	84,5	Klun et al. (2006)
Slovakia	1939	SFDT	In house test according to the method of Sabin and Feldman (1948)	4	10	Kováčová (1993)
Spain	203	MAT	Toxosreen DA test® (BioMérieux, Marcy-l'Etoile, France)	40	40,4	Mainar-Jaime and Barberan (2007)
	177	MAT	Toxosreen DA test® (BioMérieux, Marcy-l'Etoile, France)	40	57	Panadero et al. (2010)
	503	ELISA	Commercial ns	ns	49,3	García-Bocanegra et al. (2013)
Sweden	704	ELISA	In house test according to the method of Voller et al. (1976)	ns	19	Lunden et al. (1992)
Switzerland	150 adults	ELISA	ELISA according to the method of Wyss et al. (2000)	ns	80,7	Berger-Schoch et al. (2011)
	100 lambs				33	
Tunis	350	ELISA	Kit ID Screen® (ID. Vet Montpellier France)	ns	10,8	Gharbi et al. (2013)
Turkey	181	ELISA	Modification of human ELISA according to the method of Mondesire et al. (1981)	ns	31	Oncel and Vural (2006)
	95	SFDT	ns	16	49,5	Acici et al. (2008)
	152	SFDT	In house test according to method of Sabin and Feldman (1948)	16	45,4	Babur et al. (2001)
	186	SFDT	ns	16	98,92	Cicek et al. (2011)
Uruguay	1613	MAT	Toxosreen DA test® (BioMérieux, Marcy-l'Etoile, France)	64	28,7	Freyre et al. (1999)
USA	lambs 383	MAT	In house test according to the method of Dubey and Desmonts (1987)	25	27,1	Dubey et al. (2008)
Zimbabwe	23	IFA	In house IFA	50	47,8	Hove et al. (2005)

SFDT = Sabin Feldman Dye Test. ELISA = enzyme-linked immunosorbent assay. IFA = Indirect immunofluorescent antibody test. IHA = Indirect hemagglutination assay. LAT = Latex agglutination test. MAT = Modified agglutination test. PCR = Polymerase chain reaction. WB = Western blot. ns = Not specified.

The prevalence of antibodies in ewes is more than twice the prevalence in lambs, demonstrating that the seroprevalence increases with age and suggesting that lambs become infected postnatally. Seroconversion occurs in most cases before the age of four, but Dubey and Kirkbride (1989) noted that 25% of 5-year-old ewes were still seronegative in highly endemic flocks. The contamination of the environment with oocysts, the presence of cats and rodents on the pastures but also the disposal of aborted foetuses may be a risk for seronegative sheep. The risk of seroconverting was about fourfold higher on farms that left foetuses on the ground in comparison with farms that fed the aborted foetuses to dogs. Riyadh (2005) showed that burying or incineration of foetuses led to a decrease in seropositivity of sheep and goats by 8%. The seroprevalence also increased with the use of surface water as drinking water and with farm size (Vesco, 2007).

## **2.5 Economic Importance**

Toxoplasmosis has a considerable economic relevance, provided that *T. gondii* has been recognised as one of the main causes of abortion in sheep in New Zealand, Australia, The United Kingdom, Norway and the United States (Beverley et al., 1971; Buxton et al., 1991; Dubey and Welcome, 1988). Significant losses occur due to abortion if the animals become infected during pregnancy. It is difficult to accurately estimate the losses: only a small proportion of aborted lambs are handed over for diagnosis. Furthermore, samples are often examined inadequately and/or the submitted material is not even suitable for diagnosis. Moreover, existing serological tests lack specificity. In general, Toxoplasmosis does not induce clinical symptoms in ewes and since disease occurs sporadically, the farmer is not alarmed as quickly as in case of some other abortion-causing bacterial or viral infections such as foot and mouth disease or bluetongue (Dubey, 2010). In the UK it has been estimated that over 0.5 million lambs may be lost each year due to toxoplasmosis. The UK Department for Environment, Food and Rural Affairs correlated this loss with a yearly cost of €15.3 to 30.6 million to the sheep industry (Cruz-Vazquez et al., 1992; Defra, 2009).

## **2.6 Prevention of infection**

Prevention of infection relies primarily on hygienic measures to avoid infection such as absence of felids in the direct environment, direct removal of aborted fetuses, no surface water as drinking water (2.6.1). Also the use of vaccines can prevent abortion (2.6.2). Furthermore, chemotherapy or chemoprophylaxis can reduce the impact of infection (2.6.3) (Buxton et al., 1996).

### ***2.6.1 Control by protecting pregnant ewes from exposure to infection***

Outbreaks of clinical toxoplasmosis are mostly caused by exposure to oocysts (Blewett and Watson, 1983; Caballero-Ortega et al., 2008). Feed contaminated with cat faeces introduces the infection in breeding flocks. Actions should be taken to prevent the possibility of cats defecating in feedstuff such as excluding them from the farm, or at least by shielding feed storages for instance by storing it in closed bins or silos. Baling hay makes it less attractive to cats. Contamination of commercial feed cannot be excluded, but oocysts are killed between 50 and 55°C, hence bagged and pelleted feed is unlikely to be infected. Drinking water was recently discovered as a new risk factor in some countries like Brazil. Sheep drinking from running water systems had a higher risk to become infected than those drinking from still water (Andrade et al., 2013; Pinheiro et al., 2009). Another study in Ethiopia demonstrated that sheep drinking from rivers and stagnant water were more likely to become infected than animals that drank tap water (Gebremedhin et al., 2013b). Due to the higher chance of contamination with cat faeces, and thus oocysts, still water is more expected to be contaminated than running water. Anyway, water has to be taken into account as a source of infection.

### ***2.6.2 Control by vaccination***

Vaccines play an important role in the control of animal diseases, particularly in livestock. The use of a vaccine to prevent the infection of meat animals with *Toxoplasma* is desirable; in order to reduce the rate of human infection from the ingestion of undercooked infected meat. A vaccine is also required to control the adverse effects on pregnant animals.

There are three targets for a vaccination strategy:

- 1) Limitation of the acute parasitaemia and protection against congenital toxoplasmosis
- 2) Reduction of the presence of tissue cysts
- 3) Reduction of oocyst shedding by cats to limit environmental contamination

Here we restrict the discussion to a commercial vaccine used in sheep and new vaccination strategies with potential for sheep.

There is a commercial attenuated live vaccine available to protect against *Toxoplasma* abortion in sheep and goats: Ovilis<sup>®</sup> Toxovax (MSD Animal Health, New Zealand) which is allowed in the United Kingdom, Ireland, France, Spain, Portugal and New Zealand since two decades (Buxton et al., 2007). The vaccine, developed in New Zealand, consists of tachyzoites of the *T. gondii* modified strain S48 and mimics a natural infection (O'Connell et al., 1988; Wilkins et al., 1988). The S48 strain was isolated from an aborted lamb and has lost the ability to produce tissue cysts by repeated passages in mice for many years. Therefore, vaccination cannot establish a persistent infection in sheep. The vaccine has to be administered subcutaneously at least three weeks before the start of the mating period. Soon after vaccination the tachyzoites can be isolated from the blood, but no cysts are apparent in the muscles one month later. The inoculation of the S48 vaccine strain causes a short infection of 14 days, after which the parasites are cleared from the animal's system. During this period, a humoral and cellular immune response is induced, involving CD4<sup>+</sup>, CD8<sup>+</sup> T cells and IFN gamma (Buxton et al., 2007; Wastling et al., 1994; Wastling et al., 1995; Wastling et al., 1993), which protects the ewe against natural infection and against abortion for at least 18 months (Menzies, 2012). When pregnant vaccinated sheep ingest oocysts, there is a normal release of sporozoites into the gut lumen. However, the ability of the parasite to invade the gut wall followed by multiplication in mesenteric lymph nodes is inhibited and the spread via lymph and blood is prevented. Thus the pregnant uterus and the foetus are protected against the infection. In conclusion, the vaccine not only causes reduction of foetal mortality but also a reduction of tissue cysts in the dam's meat (Buxton et al., 2007; Katzer et al., 2014). So, this attenuated vaccine has the advantage that it avoids zoonotic infections as a result of consuming undercooked meat. Disadvantages of the vaccine are: (1) the possible reversion of the S48 strain to the wild type, (2) the risk for humans to become infected by handling the vaccine, (3) its short shelf-life of two to three weeks after reconstitution, and (4) the need of a cold chain during transport of the vaccine (Menzies, 2012).

The attenuated *T. gondii* strain M49 has been evaluated by Falcón and Freyre (2009) without convincing results. This illustrates that the need for a non-infectious vaccine is still relevant.

In recent years, different non-infectious vaccine candidates have been tested for inducing a protective immunity including DNA vaccines. DNA vaccination is an interesting method for the induction of specific humoral and cellular immune responses since the vaccine is very stable and once taken up by host cells can lead to major histocompatibility complex (MHC) type II presentation with activation of CD4<sup>+</sup> cells and MHC type I presentation with activation of CD8<sup>+</sup> cells, similar to live parasites. Activation of CD8<sup>+</sup> cells normally does not occur when vaccinating with protein vaccines. However, whereas DNA vaccines are very successful in mice, there is still work to improve the immune response in larger mammals such as humans and farm animals. Therefore many adjuvants have been explored in combination with DNA vaccines such as Toll-like receptor ligands and immunostimulating complexes (ISCOMs).

Research on *T. gondii* subunit vaccines consisting of one or a cocktail of antigens, has focused on the surface antigens of tachyzoites (Bhopale, 2003). The major surface antigens (SAG) are SAG1, SAG2 and SAG3. In goats, the most antigenic surface antigen is SAG1 (P30) (Conde et al., 2001). SAG1 combined to liposomes as adjuvant has been tested to immunise mice. This combination elicited a good cellular immune response in mice, resulting in a partial protection (Bulow and Boothroyd, 1991). When SAG1 was combined with adjuvants like Quil A and cholera toxin, an induction of mucosal and systemic immune responses was seen (Debard et al., 1996; Velge-Roussel et al., 2000).

Other vaccine candidates are some of the excretory/secretory antigens, which are expressed by both tachyzoites and bradyzoites (Capron and Dessaint, 1988; Cesbron-Delauw and Capron, 1993). Dense granule (GRA) molecules like GRA1 (23kDa), GRA4 (40kDa) and GRA7 (29kDa) are the leading vaccine candidates. Vaccination studies have been conducted using GRA7 formulated with different adjuvant formulations. Also, the rhoptry protein ROP2 (56kDa), expressed by tachyzoites, bradyzoites and sporozoites has been proposed as a vaccine candidate since the T-cell epitopes for the ROP2 antigen are recognised by a high percentage of immune humans (Saavedra et al., 1996).

Although many candidate vaccines have been investigated, none of them have been brought to commercial production. There is an urgent need to verify the potential of these vaccines in the target species by further experimental work and field studies.

### **2.6.3 Control by use of drugs**

Prophylactic treatment of ewes during pregnancy by monensin and sulfamezatin-pyrimethamine reduces the abortion rate significantly (Buxton et al., 1988). For monensin, the dose varies between 15 to 30 mg/animal daily. The treatment is effective but expensive (Buxton et al., 1988).

Also chemoprophylaxis with decoquinate in the diet will reduce the impact of infection. Decoquinate causes a reduction in the febrile response and in the placental damage and increases the number of lambs born and the birth weight of live lambs, in comparison with ewes not fed with decoquinate (Buxton et al., 1996).

In the cat intestine toltrazuril, a triazinetrione derivative, is very effective during the sexual replication cycle in reducing the schizogony and gametogony. This drug leads to a reduction in the multiplication rate of the parasite by e.g. killing of the tachyzoites. Swelling of the endoplasmic reticulum and visible damage to the parasite's DNA has been seen by electron microscopy. Toltrazuril causes also a reduction of the extra-intestinal developmental stages of *T. gondii* (Haberkorn, 1996). This molecule is used as treatment against avian coccidiosis and more recently against coccidiosis in calves, piglets, lambs and kids (Haberkorn, 1996; Jonsson et al., 2011; Kreiner et al., 2011; Le Sueur et al., 2009). Treatment of experimentally infected lambs with toltrazuril resulted in degenerative changes in the tissue cyst wall, resulting in an invasion of macrophages and thus reduction of the number of tissue cysts (Kul et al., 2013).

Ponazuril, another triazinetrione derivative, induces rupture of the membrane of the parasitophorous vacuole, causes mitochondrial damage in the parasites and prevents cell division, inhibiting the replication of *T. gondii* tachyzoites (Darius et al., 2004; Mitchell et al., 2004). An oral dose of 10 or 20 mg/kg ponazuril, given during 10 days, protected mice from death in experimental toxoplasmosis with RH strain (Mitchell et al., 2004).

## 2.7 References

- Acici, M., Babur, C., Kilic, S., Hokelek, M., Kurt, M., 2008. Prevalence of antibodies to *Toxoplasma gondii* infection in humans and domestic animals in Samsun province, Turkey. *Trop Anim Health Pro* 40, 311-315.
- Alvarado-Esquivel, C., Estrada-Malacon, M.A., Reyes-Hernandez, S.O., Perez-Ramirez, J.A., Trujillo-Lopez, J.I., Villena, I., Dubey, J.P., 2013a. Seroprevalence of *Toxoplasma gondii* in domestic sheep in Oaxaca State, Mexico. *J Parasitol* 99, 151-152.
- Alvarado-Esquivel, C., Garcia-Machado, C., Alvarado-Esquivel, D., Vitela-Corrales, J., Villena, I., Dubey, J.P., 2012. Seroprevalence of *Toxoplasma gondii* infection in domestic sheep in Durango State, Mexico. *J Parasitol* 98, 271-273.
- Alvarado-Esquivel, C., Silva-Aguilar, D., Villena, I., Dubey, J.P., 2013b. Seroprevalence and correlates of *Toxoplasma gondii* infection in domestic sheep in Michoacan State, Mexico. *Preventive veterinary medicine* 112, 433-437.
- Ambroise-Thomas, P., Francesio, J., Simon, J., Micouin, C., Pierson, Y., 1980. Rheumatoid factors : a cause of non-specific results in IgM anti-*toxoplasma* fluorescent tests (author's transl). *Ann Biol Clin (Paris)* 38, 315-319.
- Ambroise-Thomas, P., Garin, J.P., Rigaud, A., 1966. Improvement of the immunofluorescence technic by the use of counter-dyes. Application to *toxoplasmas*. *Presse Med* 74, 2215-2216.
- Anastasia, D., Elias, P., Nikolaos, P., Charilaos, K., Nektarios, G., 2013. *Toxoplasma gondii* and *Neospora caninum* seroprevalence in dairy sheep and goats mixed stock farming. *Vet Parasitol* 198, 387-390.
- Anderson, M.L., Barr, B.C., Conrad, P.A., 1994. Protozoal causes of reproductive failure in domestic ruminants. *Vet Clin North Am Food Anim Pract* 10, 439-461.
- Andrade, M.M., Carneiro, M., Medeiros, A.D., Neto, V.A., Vitor, R.W., 2013. Seroprevalence and risk factors associated with ovine toxoplasmosis in Northeast Brazil. *Parasite* 20, 20.
- Auer, H., Vander-Mose, A., Picher, O., Walochnik, J., Aspöck, H., 2000. Clinical and diagnostic relevance of the *Toxoplasma* IgG avidity test in the serological surveillance of pregnant women in Austria. *Parasitol Res* 86, 965-970.
- Azab, M., Beverley, J.K., 1974. Schizogony of *Toxoplasma gondii* in tissue cultures. *Z Parasitenkd* 44, 33-41.
- Babur, C., Esen, B., Biyikoglu, G., 2001. Seroprevalence of Toxoplasmosis gondii in sheep in Yozgat, Turkey. *Turk J Vet Anim Sci* 25, 283-285.
- Balfour, A.H., Fleck, D.G., Hughes, H.P., Sharp, D., 1982. Comparative study of three tests (dye test, indirect haemagglutination test, latex agglutination test) for the detection of antibodies to *Toxoplasma gondii* in human sera. *J Clin Pathol* 35, 228-232.



- Barakat, A.M.A., Abd Elazis, M.M., Fadaly, H.A., 2009. Comparative diagnosis of Toxoplasmosis in Egyptian small ruminants by Indirect Hemagglutination Assay and Elisa. *Global Veterinaria* 3, 09-14.
- Bartova, E., Sedlak, K., Literak, I., 2009. *Toxoplasma gondii* and *Neospora caninum* antibodies in sheep in the Czech Republic. *Vet Parasitol* 161, 131-132.
- Berger-Schoch, A.E., Bernet, D., Doherr, M.G., Gottstein, B., Frey, C.F., 2011. *Toxoplasma gondii* in Switzerland: a serosurvey based on meat juice analysis of slaughtered pigs, wild boar, sheep and cattle. *Zoonoses Public Health* 58, 472-478.
- Beverley, J.K., Watson, W.A., Spence, J.B., 1971. The pathology of the foetus in ovine abortion due to toxoplasmosis. *Vet Rec* 88, 174-178.
- Bhalla, M., Johnson, J.D., Holliman, R.E., Savva, D., 1999. Misdiagnosis of *toxoplasma* infection by PCR: fears unfounded. *J Clin Pathol* 52, 468-470.
- Bhopale, G.M., 2003. Development of a vaccine for toxoplasmosis: current status. *Microbes Infect* 5, 457-462.
- Blewett, D.A., Teale, A.J., Miller, J.K., Scott, G.R., Buxton, D., 1982. Toxoplasmosis in rams: possible significance of venereal transmission. *Vet Rec* 111, 73-75.
- Blewett, D.A., Watson, W.A., 1983. The epidemiology of ovine toxoplasmosis. II. Possible sources of infection in outbreaks of clinical disease. *Br Vet J* 139, 546-555.
- Bos, H.J., 1993. Development of a Live Vaccine Against Ovine Toxoplasmosis. *Toxoplasmosis, NATO ASI Series* 78, 231-243.
- Brodie, T.A., Duncan, J.L., Harvey, M.J., 1983. Role of enzootic abortion and toxoplasmosis in an outbreak of abortion in a Scottish sheep flock. *Vet Rec* 113, 437-440.
- Bulow, R., Boothroyd, J.C., 1991. Protection of mice from fatal *Toxoplasma gondii* infection by immunization with p30 antigen in liposomes. *J Immunol* 147, 3496-3500.
- Burg, J.L., Grover, C.M., Pouletty, P., Boothroyd, J.C., 1989. Direct and sensitive detection of a pathogenic protozoan, *Toxoplasma gondii*, by polymerase chain reaction. *J Clin Microbiol* 27, 1787-1792.
- Burg, J.L., Perelman, D., Kasper, L.H., Ware, P.L., Boothroyd, J.C., 1988. Molecular analysis of the gene encoding the major surface antigen of *Toxoplasma gondii*. *J Immunol* 141, 3584-3591.
- Buxton, D., 1998. Protozoan infections (*Toxoplasma gondii*, *Neospora caninum* and *Sarcocystis spp.*) in sheep and goats: recent advances. *Vet Res* 29, 289-310.
- Buxton, D., Blewett, D.A., Trees, A.J., McColgan, C., Finlayson, J., 1988. Further studies in the use of monensin in the control of experimental ovine toxoplasmosis. *J Comp Pathol* 98, 225-236.
- Buxton, D., Brebner, J., Wright, S., Maley, S.W., Thomson, K.M., Millard, K., 1996. Decoquinate and the control of experimental ovine toxoplasmosis. *Vet Rec* 138, 434-436.

- Buxton, D., Finlayson, J., 1986. Experimental infection of pregnant sheep with *Toxoplasma gondii*: pathological and immunological observations on the placenta and foetus. J Comp Pathol 96, 319-333.
- Buxton, D., Maley, S.W., Wright, S.E., Rodger, S., Bartley, P., Innes, E.A., 2007. *Toxoplasma gondii* and ovine toxoplasmosis: new aspects of an old story. Vet Parasitol 149, 25-28.
- Buxton, D., Rodger, S.M., Maley, S.W., Wright, S.E., 2006. Toxoplasmosis: The possibility of vertical transmission. Small Ruminant Res 62, 43-46.
- Buxton, D., Thomson, K., Maley, S., Wright, S., Bos, H.J., 1991. Vaccination of sheep with a live incomplete strain (S48) of *Toxoplasma gondii* and their immunity to challenge when pregnant. Vet Rec 129, 89-93.
- Caballero-Ortega, H., Palma, J.M., Garcia-Marquez, L.J., Gildo-Cardenas, A., Correa, D., 2008. Frequency and risk factors for toxoplasmosis in ovines of various regions of the State of Colima, Mexico. Parasitology 135, 1385-1389.
- Capron, A., Dessaint, J.P., 1988. Vaccination against parasitic diseases: some alternative concepts for the definition of protective antigens. Annales de l'Institut Pasteur. Immunology 139, 109-117.
- Cenci-Goga, B.T., Ciampelli, A., Sechi, P., Veronesi, F., Moretta, I., Cambiotti, V., Thompson, P.N., 2013. Seroprevalence and risk factors for *Toxoplasma gondii* in sheep in Grosseto district, Tuscany, Italy. BMC Vet Res 9, 25.
- Cesbron-Delauw, M.F., Capron, A., 1993. Excreted/secreted antigens of *Toxoplasma gondii* - their origin and role in the host-parasite interaction. Research in immunology 144, 41-44.
- Chabbert, E., Lachaud, L., Crobu, L., Bastien, P., 2004. Comparison of two widely used PCR primer systems for detection of *toxoplasma* in amniotic fluid, blood, and tissues. J Clin Microbiol 42, 1719-1722.
- Chikweto, A., Kumthekar, S., Tiwari, K., Nyack, B., Deokar, M.S., Stratton, G., Macpherson, C.N., Sharma, R.N., Dubey, J.P., 2011. Seroprevalence of *Toxoplasma gondii* in pigs, sheep, goats, and cattle from Grenada and Carriacou, West Indies. J Parasitol 97, 950-951.
- Cicek, H., Babur, C., Eser, M., 2011. Seroprevalence of *Toxoplasma gondii* in Pirlak sheep in the Afyonkarahisar Province of Turkey. Turkiye parazitolojii dergisi / Turkiye Parazitoloji Dernegi = Acta parasitologica Turcica / Turkish Society for Parasitology 35, 137-139.
- Clementino, M.M., Souza, M.F., Andrade Neto, V.F., 2007. Seroprevalence and *Toxoplasma gondii*-IgG avidity in sheep from Lajes, Brazil. Vet Parasitol 146, 199-203.
- Conde, M., Caballero, J.M., Rodriguez-Ponce, E., Ruiz, A., Gonzalez, J., 2001. Analysis of IgG response to experimental infection with RH *Toxoplasma gondii* in goats. Comp Immunol Microbiol Infect Dis 24, 197-206.
- Cruz-Vazquez, C., Garcia-Vazquez, Z., Rosario-Cruz, R., Solorzano-Salgado, M., 1992. Ovine toxoplasmosis in Huitzilac, Morelos, Mexico. Prev. Vet. Med. 12, 27-33.

- Darius, A.K., Mehlhorn, H., Heydorn, A.O., 2004. Effects of toltrazuril and ponazuril on the fine structure and multiplication of tachyzoites of the NC-1 strain of *Neospora caninum* (a synonym of *Hammondia heydorni*) in cell cultures. *Parasitol Res* 92, 453-458.
- Debard, N., Buzoni-Gatel, D., Bout, D., 1996. Intranasal immunization with SAG1 protein of *Toxoplasma gondii* in association with cholera toxin dramatically reduces development of cerebral cysts after oral infection. *Infect Immun* 64, 2158-2166.
- Deconinck, P., Pangui, L.J., Akakpo, J., Garrouste, A., Ouattara, L., Roger, F., Tibayrenc, R., Dorchies, P., 1996. Prévalence de la toxoplasmose chez les petits ruminants en Afrique tropicale: résultats d'une enquête séro-épidémiologique sur 1042 animaux. *Rev. Méd. Vét.* 147, 377-378.
- Defra 2009. Summary profile for toxoplasmosis.
- Dempster, R.P., Wilkins, M., Green, R.S., de Lisle, G.W., 2011. Serological survey of *Toxoplasma gondii* and *Campylobacter fetus* in sheep from New Zealand. *N Z Vet J* 59, 155-159.
- Denkers, E.Y., Gazzinelli, R.T., 1998. Regulation and function of T-cell-mediated immunity during *Toxoplasma gondii* infection. *Clin Microbiol Rev* 11, 569-588.
- Derouin, F., Mazon, M.C., Garin, Y.J., 1987. Comparative study of tissue culture and mouse inoculation methods for demonstration of *Toxoplasma gondii*. *J Clin Microbiol* 25, 1597-1600.
- Desmonts, G., Remington, J.S., 1980. Direct agglutination test for diagnosis of *Toxoplasma* infection: method for increasing sensitivity and specificity. *J Clin Microbiol* 11, 562-568.
- Dubey, J.P., 1988. Long-term persistence of *Toxoplasma gondii* in tissues of pigs inoculated with *T. gondii* oocysts and effect of freezing on viability of tissue cysts in pork. *Am J Vet Res* 49, 910-913.
- Dubey, J.P., 2001. Oocyst shedding by cats fed isolated bradyzoites and comparison of infectivity of bradyzoites of the VEG strain *Toxoplasma gondii* to cats and mice. *J Parasitol* 87, 215-219.
- Dubey, J.P., 2006. Comparative infectivity of oocysts and bradyzoites of *Toxoplasma gondii* for intermediate (mice) and definitive (cats) hosts. *Vet Parasitol* 140, 69-75.
- Dubey, J.P., 2009. Toxoplasmosis in sheep--the last 20 years. *Vet Parasitol* 163, 1-14.
- Dubey, J.P., 2010. *Toxoplasmosis of Animals and Humans*. (2nd ed.) CRC Press, Boca Raton, FL 33487, USA, 313 p.
- Dubey, J.P., Emond, J.P., Desmonts, G., Anderson, W.R., 1987. Serodiagnosis of postnatally and prenatally induced toxoplasmosis in sheep. *Am J Vet Res* 48, 1239-1243.
- Dubey, J.P., Frenkel, J.K., 1972. Cyst-induced toxoplasmosis in cats. *J Protozool* 19, 155-177.
- Dubey, J.P., Kirkbride, C.A., 1989. Enzootic toxoplasmosis in sheep in north-central United States. *J Parasitol* 75, 673-676.

- Dubey, J.P., Lindsay, D.S., Speer, C.A., 1998. Structures of *Toxoplasma gondii* tachyzoites, bradyzoites, and sporozoites and biology and development of tissue cysts. *Clin Microbiol Rev* 11, 267-299.
- Dubey, J.P., Miller, N.L., Frenkel, J.K., 1970. The *Toxoplasma gondii* oocyst from cat feces. *J Exp Med* 132, 636-662.
- Dubey, J.P., Sharma, S.P., 1980. Parasitemia and tissue infection in sheep fed *Toxoplasma gondii* oocysts. *J Parasitol* 66, 111-114.
- Dubey, J.P., Sundar, N., Hill, D., Velmurugan, G.V., Bandini, L.A., Kwok, O.C.H., Majumdar, D., Su, C., 2008. High prevalence and abundant atypical genotypes of *Toxoplasma gondii* isolated from lambs destined for human consumption in the USA. *International Journal for Parasitology* 38, 999-1006.
- Dubey, J.P., Thulliez, P., Weigel, R.M., Andrews, C.D., Lind, P., Powell, E.C., 1995. Sensitivity and specificity of various serologic tests for detection of *Toxoplasma gondii* infection in naturally infected sows. *Am J Vet Res* 56, 1030-1036.
- Dubey, J.P., Welcome, F.L., 1988. *Toxoplasma gondii*-induced abortion in sheep. *J Am Vet Med Assoc* 193, 697-700.
- Dubey, J.P.a.B., C. P., 1988b. *Toxoplasmosis of animals and man*. CRC Press, Inc., Boca Raton, FL 33431, USA, 220 p.
- Dumetre, A., Ajzenberg, D., Rozette, L., Mercier, A., Darde, M.L., 2006. *Toxoplasma gondii* infection in sheep from Haute-Vienne, France: seroprevalence and isolate genotyping by microsatellite analysis. *Vet Parasitol* 142, 376-379.
- Dumetre, A., Darde, M.L., 2004. Purification of *Toxoplasma gondii* oocysts by cesium chloride gradient. *Journal of microbiological methods* 56, 427-430.
- Duncanson, P., Terry, R.S., Smith, J.E., Hide, G., 2001. High levels of congenital transmission of *Toxoplasma gondii* in a commercial sheep flock. *Int J Parasitol* 31, 1699-1703.
- Dupon, M., Cazenave, J., Pellegrin, J.L., Ragnaud, J.M., Cheyrou, A., Fischer, I., Leng, B., Lacut, J.Y., 1995. Detection of *Toxoplasma gondii* by PCR and tissue culture in cerebrospinal fluid and blood of human immunodeficiency virus-seropositive patients. *J Clin Microbiol* 33, 2421-2426.
- Edelhofer, R., Aspöck, H., 1996. Infektionsquellen und Infektionswege aus der Sicht des Toxoplasmose-Screenings der Schwangeren in Österreich. *Mitt. Österr. Ges. Tropenmed. Parasitol.* 18, 59-70.
- Ellis, J.T., 1998. Polymerase chain reaction approaches for the detection of *Neospora caninum* and *Toxoplasma gondii*. *Int J Parasitol* 28, 1053-1060.
- Ely, K.H., Kasper, L.H., Khan, I.A., 1999. Augmentation of the CD8+ T cell response by IFN-gamma in IL-12-deficient mice during *Toxoplasma gondii* infection. *J Immunol* 162, 5449-5454.

- Eyles, D.E., Coleman, N., 1956. Relationship of size of inoculum to time to death in mice infected with *Toxoplasma gondii*. J Parasitol 42, 272-276.
- Falcon, J., Freyre, A., 2009. *Toxoplasma gondii*: prototype immunization of lambs against formation of muscle and brain cysts. Vet Parasitol 166, 15-20.
- Fallahi, S., Seyyed Tabaei, S.J., Pournia, Y., Zebardast, N., Kazemi, B., 2014. Comparison of loop-mediated isothermal amplification (LAMP) and nested-PCR assay targeting the RE and B1 gene for detection of *Toxoplasma gondii* in blood samples of children with leukaemia. Diagn Microbiol Infect Dis 79, 347-354.
- Ferguson, D.J., 2009. *Toxoplasma gondii*: 1908-2008, homage to Nicolle, Manceaux and Splendore. Mem Inst Oswaldo Cruz 104, 133-148.
- Ferguson, D.J., Hutchison, W.M., Siim, J.C., 1975. The ultrastructural development of the macrogamete and formation of the oocyst wall of *Toxoplasma gondii*. Acta pathologica et microbiologica Scandinavica. Section B, Microbiology 83, 491-505.
- Fraga, D., Meulia, T., Fenster, S., 2014. Real-Time PCR. Current Protocols Essential Laboratory Techniques. 10:10.13:10.13.11–10.13.40.
- Fredriksson, G., Buxton, D., Ugglä, A., Kindahl, H., Edqvist, L.E., 1990. The effect of *Toxoplasma gondii* infection in unvaccinated and iscom-vaccinated pregnant ewes as monitored by plasma levels of 15-ketodihydroprostaglandin F2 alpha, progesterone, and oestrone sulphate. Zentralbl Veterinärmed A 37, 113-122.
- Frenkel, J.K., 1967. Adoptive immunity to intracellular infection. J Immunol 98, 1309-1319.
- Freyre, A., Bonino, J., Falcon, J., Castells, D., Correa, O., Casaretto, A., 1999. The incidence and economic significance of ovine toxoplasmosis in Uruguay. Vet Parasitol 81, 85-88.
- Fuccillo, D.A., Madden, D.L., Tzan, N., Sever, J.L., 1987. Difficulties associated with serological diagnosis of *Toxoplasma gondii* infections. Diagnostic and clinical immunology 5, 8-13.
- Fuentes, I., Rodriguez, M., Domingo, C.J., del Castillo, F., Juncosa, T., Alvar, J., 1996. Urine sample used for congenital toxoplasmosis diagnosis by PCR. J Clin Microbiol 34, 2368-2371.
- Fulton, J.D., Turk, J.L., 1959. Direct agglutination test for *Toxoplasma gondii*. Lancet 2, 1068-1069.
- Fusco, G., Rinaldi, L., Guarino, A., Proroga, Y.T., Pesce, A., Giuseppina de, M., Cringoli, G., 2007. *Toxoplasma gondii* in sheep from the Campania region (Italy). Vet Parasitol 149, 271-274.
- Gaffuri, A., Giacometti, M., Tranquillo, V.M., Magnino, S., Cordioli, P., Lanfranchi, P., 2006. Serosurvey of roe deer, chamois and domestic sheep in the central Italian Alps. J Wildl Dis 42, 685-690.
- Garcia-Bocanegra, I., Cabezon, O., Hernandez, E., Martinez-Cruz, M.S., Martinez-Moreno, A., Martinez-Moreno, J., 2013. *Toxoplasma gondii* in ruminant species (cattle, sheep, and goats) from southern Spain. J Parasitol 99, 438-440.

- Gazzinelli, R.T., Denkers, E.Y., Sher, A., 1993a. Host resistance to *Toxoplasma gondii*: model for studying the selective induction of cell-mediated immunity by intracellular parasites. *Infectious agents and disease* 2, 139-149.
- Gazzinelli, R.T., Hakim, F.T., Hieny, S., Shearer, G.M., Sher, A., 1991. Synergistic role of CD4+ and CD8+ T lymphocytes in IFN-gamma production and protective immunity induced by an attenuated *Toxoplasma gondii* vaccine. *J Immunol* 146, 286-292.
- Gazzinelli, R.T., Hieny, S., Wynn, T.A., Wolf, S., Sher, A., 1993b. Interleukin 12 is required for the T-lymphocyte-independent induction of interferon gamma by an intracellular parasite and induces resistance in T-cell-deficient hosts. *Proc Natl Acad Sci U S A* 90, 6115-6119.
- Gebremedhin, E.Z., Agonafir, A., Tessema, T.S., Tilahun, G., Medhin, G., Vitale, M., Di Marco, V., 2013b. Some risk factors for reproductive failures and contribution of *Toxoplasma gondii* infection in sheep and goats of Central Ethiopia: A cross-sectional study. *Res Vet Sci* 95, 894-900.
- Gebremedhin, E.Z., Agonafir, A., Tessema, T.S., Tilahun, G., Medhin, G., Vitale, M., Di Marco, V., Cox, E., Vercruysse, J., Dorny, P., 2013a. Seroepidemiological study of ovine toxoplasmosis in East and West Shewa Zones of Oromia Regional State, Central Ethiopia. *BMC Vet Res* 9, 117.
- Gharbi, M., Zribi, L., Jedidi, M., Chakkhari, H., Hamdi, S., R'Hayem, S., Zribi, N., Souli, M., Darghouth, M.A., 2013. Prevalence of *Toxoplasma gondii* infection in Tunisian sheep. *Bull Soc Pathol Exot* 106, 184-187.
- Ghoneim, N.H., Shalaby, S.I., Hassanain, N.A., Zeedan, G.S., Soliman, Y.A., Abdalhamed, A.M., 2010. Comparative study between serological and molecular methods for diagnosis of toxoplasmosis in women and small ruminants in Egypt. *Foodborne pathogens and disease* 7, 17-22.
- Giangaspero, M., Bonfini, B., Orusa, R., Savini, G., Osawa, T., Harasawa, R., 2013. Epidemiological survey for *Toxoplasma gondii*, *Chlamydiopsittaci* var. *ovis*, *Mycobacterium paratuberculosis*, *Coxiella burnetii*, *Brucella* spp., leptospirosis and *Orf* virus among sheep from northern districts of Japan. *The Journal of veterinary medical science / the Japanese Society of Veterinary Science* 75, 679-684.
- Gordon, M.A., Duncan, R.A., Kingsley, L.C., 1981. Automated immunofluorescence test for toxoplasmosis. *J Clin Microbiol* 13, 283-285.
- Gorecki, M.T., Andrzejewska, I., Steppa, R., 2008. Is order of voluntarily entrance to milking parlour related to *Toxoplasma gondii* infection in sheep - A brief note. *Appl Anim Behav Sci* 110, 392-396.
- Gorman, T., Arancibia, J.P., Lorca, M., Hird, D., Alcaino, H., 1999. Seroprevalence of *Toxoplasma gondii* infection in sheep and alpacas (*Llama pacos*) in Chile. *Preventive veterinary medicine* 40, 143-149.

- Guimaraes, L.A., Bezerra, R.A., Rocha Dde, S., Albuquerque, G.R., 2013. Prevalence and risk factors associated with anti-*Toxoplasma gondii* antibodies in sheep from Bahia state, Brazil. *Revista brasileira de parasitologia veterinaria = Brazilian journal of veterinary parasitology : Orgao Oficial do Colegio Brasileiro de Parasitologia Veterinaria* 22, 220-224.
- Gustafson, P.V., Agar, H.D., Cramer, D.I., 1954. An electron microscope study of *Toxoplasma*. *Am J Trop Med Hyg* 3, 1008-1022.
- Haberkorn, A., 1996. Chemotherapy of human and animal coccidiosis: state and perspectives. *Parasitol Res* 82, 193-199.
- Halova, D., Mulcahy, G., Rafter, P., Turcekova, L., Grant, T., de Waal, T., 2013. *Toxoplasma gondii* in Ireland: seroprevalence and novel molecular detection method in sheep, pigs, deer and chickens. *Zoonoses Public Health* 60, 168-173.
- Hamidinejat, H., Gorannejad, S., Ghorbanpoor, M., Nabavi, L., Akbarnejad, F., 2008. Role of *Toxoplasma gondii* in abortion of ewes in Ahvaz (South-West Iran). *B Vet I Pulawy* 52, 369-371.
- Hartley, W.J., Jebson, J. L., McFarlane, D., 1954. New Zealand Type II abortions in ewes. *Australian Veterinary Journal* 30, 216-218.
- Hartley, W.J., Moyle, G.G., 1974. Further observations on the epidemiology of ovine *Toxoplasma* infection. *Aust J Exp Biol Med Sci* 52, 647-653.
- Hecker, Y.P., Moore, D.P., Manazza, J.A., Unzaga, J.M., Spath, E.J., Pardini, L.L., Venturini, M.C., Roberi, J.L., Campero, C.M., 2013. First report of seroprevalence of *Toxoplasma gondii* and *Neospora caninum* in dairy sheep from Humid Pampa, Argentina. *Trop Anim Health Prod* 45, 1645-1647.
- Hedman, K., Lappalainen, M., Seppä, I., Makela, O., 1989. Recent primary *toxoplasma* infection indicated by a low avidity of specific IgG. *J Infect Dis* 159, 736-740.
- Hitt, J.A., Filice, G.A., 1992. Detection of *Toxoplasma gondii* parasitemia by gene amplification, cell culture, and mouse inoculation. *J Clin Microbiol* 30, 3181-3184.
- Homan, W.L., Vercammen, M., De Braekeleer, J., Verschueren, H., 2000. Identification of a 200- to 300-fold repetitive 529 bp DNA fragment in *Toxoplasma gondii*, and its use for diagnostic and quantitative PCR. *Int J Parasitol* 30, 69-75.
- Hove, T., Lind, P., Mukaratirwa, S., 2005. Seroprevalence of *Toxoplasma gondii* infection in goats and sheep in Zimbabwe. *Onderstepoort J Vet* 72, 267-272.
- Hughes, H.P., Hudson, L., Fleck, D.G., 1986. In vitro culture of *Toxoplasma gondii* in primary and established cell lines. *Int J Parasitol* 16, 317-322.
- Hutchinson, J.P., Wear, A.R., Lambton, S.L., Smith, R.P., Pritchard, G.C., 2011. Survey to determine the seroprevalence of *Toxoplasma gondii* infection in British sheep flocks. *Vet Rec* 169, 582.

- Innes, E.A., Bartley, P.M., Buxton, D., Katzer, F., 2009b. Ovine toxoplasmosis. *Parasitology* 136, 1887-1894.
- Innes, E.A., Bartley, P.M., Maley, S.W., Wright, S.E., Buxton, D., 2007. Comparative host-parasite relationships in ovine toxoplasmosis and bovine neosporosis and strategies for vaccination. *Vaccine* 25, 5495-5503.
- Innes, E.A., Panton, W.R., Sanderson, A., Thomson, K.M., Wastling, J.M., Maley, S., Buxton, D., 1995a. Induction of CD4+ and CD8+ T cell responses in efferent lymph responding to *Toxoplasma gondii* infection: analysis of phenotype and function. *Parasite Immunol* 17, 151-160.
- Innes, E.A., Panton, W.R., Thomson, K.M., Maley, S., Buxton, D., 1995b. Kinetics of interferon gamma production in vivo during infection with the S48 vaccine strain of *Toxoplasma gondii*. *J Comp Pathol* 113, 89-94.
- Innes, E.A., Vermeulen, A.N., 2006. Vaccination as a control strategy against the coccidial parasites *Eimeria*, *Toxoplasma* and *Neospora*. *Parasitology* 133 Suppl, S145-168.
- Innes, E.A., Wright, S., Bartley, P., Maley, S., Macaldowie, C., Esteban-Redondo, I., Buxton, D., 2005. The host-parasite relationship in bovine neosporosis. *Vet Immunol Immunopathol* 108, 29-36.
- Jacobs, L., Lunde, M.N., 1957. A hemagglutination test for toxoplasmosis. *J Parasitol* 43, 308-314.
- Jacobs, L., Melton, M.L., 1954. Modifications in virulence of a strain of *Toxoplasma gondii* by passage in various hosts. *Am J Trop Med Hyg* 3, 447-457.
- Jankû, J., 1923. Pathogens a pathologica anatomie T. Zv. vrozaneho kolobou zlute skurney oku normal ne velikem a mikrophthalmickem s nalezem parisitu v sitnici. *Cas Lek Ses* 62, 1021-1027.
- Jauregui, L.H., Higgins, J., Zarlenga, D., Dubey, J.P., Lunney, J.K., 2001. Development of a real-time PCR assay for detection of *Toxoplasma gondii* in pig and mouse tissues. *J Clin Microbiol* 39, 2065-2071.
- Jenum, P.A., Stray-Pedersen, B., Gundersen, A.G., 1997. Improved diagnosis of primary *Toxoplasma gondii* infection in early pregnancy by determination of anti-toxoplasma immunoglobulin G avidity. *J Clin Microbiol* 35, 1972-1977.
- Johnson, A.M., 1984. Strain-dependent, route of challenge-dependent, murine susceptibility to toxoplasmosis. *Z Parasitenkd* 70, 303-309.
- Jokelainen, P., Nareaho, A., Knaapi, S., Oksanen, A., Rikula, U., Sukura, A., 2010. *Toxoplasma gondii* in wild cervids and sheep in Finland: north-south gradient in seroprevalence. *Vet Parasitol* 171, 331-336.
- Jonsson, N.N., Piper, E.K., Gray, C.P., Deniz, A., Constantinoiu, C.C., 2011. Efficacy of toltrazuril 5 % suspension against *Eimeria bovis* and *Eimeria zuernii* in calves and observations on the associated immunopathology. *Parasitol Res* 109 Suppl 1, S113-128.



- Joynson, D.H., Payne, R.A., Rawal, B.K., 1990. Potential role of IgG avidity for diagnosing toxoplasmosis. *J Clin Pathol* 43, 1032-1033.
- Kamani, J., Mani, A.U., Egwu, G.O., 2010. Seroprevalence of *Toxoplasma gondii* infection in domestic sheep and goats in Borno state, Nigeria. *Trop Anim Health Prod* 42, 793-797.
- Kantzoura, V., Diakou, A., Kouam, M.K., Feidas, H., Theodoropoulou, H., Theodoropoulos, G., 2013. Seroprevalence and risk factors associated with zoonotic parasitic infections in small ruminants in the Greek temperate environment. *Parasitol Int* 62, 554-560.
- Katzer, F., Brulisauer, F., Collantes-Fernandez, E., Bartley, P.M., Burrells, A., Gunn, G., Maley, S.W., Cousens, C., Innes, E.A., 2011. Increased *Toxoplasma gondii* positivity relative to age in 125 Scottish sheep flocks; evidence of frequent acquired infection. *Vet Res* 42, 121.
- Kervrann, C., Trubuil, A., Hoebeke, M., Kiêu, K., Istas, J., Rospars, J.P., 2000. Analyse d'images en microscopie multidimensionnelle. 1-34.
- Khan, I.A., Ely, K.H., Kasper, L.H., 1994. Antigen-specific CD8+ T cell clone protects against acute *Toxoplasma gondii* infection in mice. *J Immunol* 152, 1856-1860.
- Kirisits, M.J., Mui, E., McLeod, R., 2000. Measurement of the efficacy of vaccines and antimicrobial therapy against infection with *Toxoplasma gondii*. *Int J Parasitol* 30, 149-155.
- Klun, I., Djurkovic-Djakovic, O., Katic-Radivojevic, S., Nikolic, A., 2006. Cross-sectional survey on *Toxoplasma gondii* infection in cattle, sheep and pigs in Serbia: seroprevalence and risk factors. *Vet Parasitol* 135, 121-131.
- Kong, Q.M., Lu, S.H., Tong, Q.B., Lou, D., Chen, R., Zheng, B., Kumagai, T., Wen, L.Y., Ohta, N., Zhou, X.N., 2012. Loop-mediated isothermal amplification (LAMP): early detection of *Toxoplasma gondii* infection in mice. *Parasites & vectors* 5, 2.
- Konishi, E., 1987. A pregnant woman with a high level of naturally occurring immunoglobulin M antibodies to *Toxoplasma gondii*. *Am J Obstet Gynecol* 157, 832-833.
- Kourenti, C., Heckerroth, A., Tenter, A., Karanis, P., 2003. Development and application of different methods for the detection of *Toxoplasma gondii* in water. *Appl Environ Microbiol* 69, 102-106.
- Kováčová, D., 1993. Sérologická prevalence *Toxoplasma gondii* u oviec. *Veterinarstvi* 43, 51-54.
- Kreiner, T., Worliczek, H.L., Tichy, A., Joachim, A., 2011. Influence of toltrazuril treatment on parasitological parameters and health performance of piglets in the field--an Austrian experience. *Vet Parasitol* 183, 14-20.
- Kul, O., Yildiz, K., Ocal, N., Freyre, A., Deniz, A., Karahan, S., Atmaca, H.T., Gokpinar, S., Dincel, G.C., Uzunalioglu, T., Terzi, O.S., 2013. In-vivo efficacy of toltrazuril on experimentally induced *Toxoplasma gondii* tissue cysts in lambs: a novel strategy for prevention of human exposure to meat-borne toxoplasmosis. *Res Vet Sci* 94, 269-276.

- Langoni, H., Greca, H., Jr., Guimaraes, F.F., Ullmann, L.S., Gaio, F.C., Uehara, R.S., Rosa, E.P., Amorim, R.M., Da Silva, R.C., 2011. Serological profile of *Toxoplasma gondii* and *Neospora caninum* infection in commercial sheep from Sao Paulo State, Brazil. *Vet Parasitol* 177, 50-54.
- Le Sueur, C., Mage, C., Mundt, H.C., 2009. Efficacy of toltrazuril (Baycox 5% suspension) in natural infections with pathogenic *Eimeria spp.* in housed lambs. *Parasitol Res* 104, 1157-1162.
- Liesenfeld, O., Press, C., Montoya, J.G., Gill, R., Isaac-Renton, J.L., Hedman, K., Remington, J.S., 1997. False-positive results in immunoglobulin M (IgM) *toxoplasma* antibody tests and importance of confirmatory testing: the Platelia Toxo IgM test. *J Clin Microbiol* 35, 174-178.
- Liesenfeld, O., Roth, A., Weinke, T., Foss, H.D., Hahn, H., 1994. A case of disseminated toxoplasmosis-value of PCR for the diagnosis. *J Infect* 29, 133-138.
- Lindquist, H.D., Bennett, J.W., Hester, J.D., Ware, M.W., Dubey, J.P., Everson, W.V., 2003. Autofluorescence of *Toxoplasma gondii* and related coccidian oocysts. *J Parasitol* 89, 865-867.
- Liu, Q., Ma, R., Zhao, Q., Shang, L., Cai, J., Wang, X., Li, J., Hu, G., Jin, H., Gao, H., 2010. Seroprevalence of *Toxoplasma gondii* infection in Tibetan sheep in northwestern China. *J Parasitol* 96, 1222-1223.
- Lopes, A.P., Dubey, J.P., Neto, F., Rodrigues, A., Martins, T., Rodrigues, M., Cardoso, L., 2013. Seroprevalence of *Toxoplasma gondii* infection in cattle, sheep, goats and pigs from the North of Portugal for human consumption. *Vet Parasitol* 193, 266-269.
- Lunde, M.N., Jacobs, L., 1959. Characteristics of the *Toxoplasma* hemagglutination test antigen. *J Immunol* 82, 146-150.
- Lunden, A., Carlsson, U., Naslund, K., 1992. Toxoplasmosis and border disease in 54 Swedish sheep flocks. Seroprevalence and incidence during one gestation period. *Acta veterinaria Scandinavica* 33, 175-184.
- Luo, W., Aosai, F., Ueda, M., Yamashita, K., Shimizu, K., Sekiya, S., Yano, A., 1997. Kinetics in parasite abundance in susceptible and resistant mice infected with an avirulent strain of *Toxoplasma gondii* by using quantitative competitive PCR. *J Parasitol* 83, 1070-1074.
- MacPherson, J.M., Gajadhar, A.A., 1993. Sensitive and specific polymerase chain reaction detection of *Toxoplasma gondii* for veterinary and medical diagnosis. *Can J Vet Res* 57, 45-48.
- Mainar-Jaime, R.C., Barberan, M., 2007. Evaluation of the diagnostic accuracy of the modified agglutination test (MAT) and an indirect ELISA for the detection of serum antibodies against *Toxoplasma gondii* in sheep through Bayesian approaches. *Vet Parasitol* 148, 122-129.
- Masala, G., Porcu, R., Madau, L., Tanda, A., Ibba, B., Satta, G., Tola, S., 2003. Survey of ovine and caprine toxoplasmosis by IFAT and PCR assays in Sardinia, Italy. *Vet Parasitol* 117, 15-21.
- Mason, S., Quinnell, R.J., Smith, J.E., 2010. Detection of *Toxoplasma gondii* in lambs via PCR screening and serological follow-up. *Vet Parasitol* 169, 258-263.

- McColgan, C., Buxton, D., Blewett, D.A., 1988. Titration of *Toxoplasma gondii* oocysts in non-pregnant sheep and the effects of subsequent challenge during pregnancy. *Vet Rec* 123, 467-470.
- McGovern, K.E., Wilson, E.H., 2013. Dark side illuminated: imaging of *Toxoplasma gondii* through the decades. *Parasites & vectors* 6, 334.
- Melzer, T.C., Cranston, H.J., Weiss, L.M., Halonen, S.K., 2010. Host Cell Preference of *Toxoplasma gondii* Cysts in Murine Brain: A Confocal Study. *Journal of neuroparasitology* 1.
- Mendonca, C.E., Barros, S.L., Guimaraes, V.A., Ferraudo, A.S., Munhoz, A.D., 2013. Prevalence and risk factors associated to ovine toxoplasmosis in northeastern Brazil. *Revista brasileira de parasitologia veterinaria = Brazilian journal of veterinary parasitology : Orgao Oficial do Colegio Brasileiro de Parasitologia Veterinaria* 22, 230-234.
- Menzies, P.I., 2012. Vaccination programs for reproductive disorders of small ruminants. *Anim Reprod Sci* 130, 162-172.
- Mitchell, S.M., Zajac, A.M., Davis, W.L., Lindsay, D.S., 2004. Efficacy of ponazuril in vitro and in preventing and treating *Toxoplasma gondii* infections in mice. *J Parasitol* 90, 639-642.
- Montoya, A., Miro, G., Mateo, M., Ramirez, C., Fuentes, I., 2009. Detection of *Toxoplasma gondii* in cats by comparing bioassay in mice and polymerase chain reaction (PCR). *Vet Parasitol* 160, 159-162.
- Morley, E.K., Williams, R.H., Hughes, J.M., Terry, R.S., Duncanson, P., Smith, J.E., Hide, G., 2005. Significant familial differences in the frequency of abortion and *Toxoplasma gondii* infection within a flock of Charollais sheep. *Parasitology* 131, 181-185.
- Morley, E.K., Williams, R.H., Hughes, J.M., Thomasson, D., Terry, R.S., Duncanson, P., Smith, J.E., Hide, G., 2008. Evidence that primary infection of Charollais sheep with *Toxoplasma gondii* may not prevent foetal infection and abortion in subsequent lambings. *Parasitology* 135, 169-173.
- Naot, Y., Remington, J.S., 1980. An enzyme-linked immunosorbent assay for detection of IgM antibodies to *Toxoplasma gondii*: use for diagnosis of acute acquired toxoplasmosis. *J Infect Dis* 142, 757-766.
- Negash, T., Tilahun, G., Patton, S., Prevot, F., Dorchie, P., 2004. Serological survey on Toxoplasmosis in sheep and goats in Nazareth, Ethiopia. *Rev Med Vet-Toulouse* 155, 486-487.
- Nery-Guimaraes, F., Venancio, I.A., Lage, H.A., Grynberg, N.F., 1968. Diagnosis of "*toxoplasma*" in tissue using the immunofluorescence technic. *Hospital* 74, 1943-1948.
- O'Connell, E., Wilkins, M.F., Te Punga, W.A., 1988. Toxoplasmosis in sheep. II. The ability of a live vaccine to prevent lamb losses after an intravenous challenge with *Toxoplasma gondii*. *N Z Vet J* 36, 1-4.

- Oncel, T., Vural, G., 2006. Occurrence of *Toxoplasma gondii* antibodies in sheep in Istanbul, Turkey. *Veterinarski Arhiv.* 76, 547-553.
- Opsteegh, M., Langelaar, M., Sprong, H., den Hartog, L., De Craeye, S., Bokken, G., Ajzenberg, D., Kijlstra, A., van der Giessen, J., 2010a. Direct detection and genotyping of *Toxoplasma gondii* in meat samples using magnetic capture and PCR. *Int J Food Microbiol* 139, 193-201.
- Opsteegh, M., Teunis, P., Mensink, M., Zuchner, L., Titilincu, A., Langelaar, M., van der Giessen, J., 2010b. Evaluation of ELISA test characteristics and estimation of *Toxoplasma gondii* seroprevalence in Dutch sheep using mixture models. *Preventive veterinary medicine* 96, 232-240.
- Orlando, C., Pinzani, P., Pazzagli, M., 1998. Developments in quantitative PCR. *Clin Chem Lab Med* 36, 255-269.
- Oura, C.A., Innes, E.A., Wastling, J.M., Entrican, G., Panton, W.R., 1993. The inhibitory effect of ovine recombinant interferon-gamma on intracellular replication of *Toxoplasma gondii*. *Parasite Immunol* 15, 535-538.
- Owen, M.R., Clarkson, M.J., Trees, A.J., 1998. Acute phase *toxoplasma* abortions in sheep. *Vet Rec* 142, 480-482.
- Palosuo, T., Aho, K., 1983. Technical falsely positive rheumatoid factor by ELISA in sera with elevated IgM levels. *Medical biology* 61, 203-207.
- Panadero, R., Paineira, A., Lopez, C., Vazquez, L., Paz, A., Diaz, P., Dacal, V., Cienfuegos, S., Fernandez, G., Lago, N., Diez-Banos, P., Morrondo, P., 2010. Seroprevalence of *Toxoplasma gondii* and *Neospora caninum* in wild and domestic ruminants sharing pastures in Galicia (Northwest Spain). *Res Vet Sci* 88, 111-115.
- Pinheiro, J.W., Jr., Mota, R.A., Oliveira, A.A., Faria, E.B., Gondim, L.F., da Silva, A.V., Anderlini, G.A., 2009. Prevalence and risk factors associated to infection by *Toxoplasma gondii* in ovine in the State of Alagoas, Brazil. *Parasitol Res* 105, 709-715.
- Plant, J.W., Richardson, N., Moyle, G.G., 1974. *Toxoplasma* infection and abortion in sheep associated with feeding of grain contaminated with cat faeces. *Aust Vet J* 50, 19-21.
- Prelezov, P., Koinarski, V., Georgieva, D., 2008. Seroprevalence of *Toxoplasma gondii* infection among sheep and goats in the Stara Zagora region. *Bulgarian. J. Vet. Med.* 11, 113-119.
- Pujol-Rique, M., Derouin, F., Garcia-Quintanilla, A., Valls, M.E., Miro, J.M., de Anta, M.T.J., 1999. Design of a one-tube hemi-nested PCR for detection of *Toxoplasma gondii* and comparison of three DNA purification methods. *Journal of Medical Microbiology* 48, 857-862.
- Raeghi, S., Akaberi, A., Sedeghi, S., 2011. Seroprevalence of *Toxoplasma gondii* in Sheep, Cattle and Horses in Urmia North-West of Iran. *Iranian journal of parasitology* 6, 90-94.
- Ramzan, M., Akhtar, M., Muhammad, F., Hussain, I., Hiszczynska-Sawicka, E., Haq, A.U., Mahmood, M.S., Hafeez, M.A., 2009. Seroprevalence of *Toxoplasma gondii* in sheep and goats in Rahim Yar Khan (Punjab), Pakistan. *Trop Anim Health Prod* 41, 1225-1229.

- Reif, J.S., Samame, H.A., Ameghino, E., Lopezniето, E., Demartini, J.C., 1989. Adverse Reproductive Outcome and Antibody to *Toxoplasma Gondii* in a Cohort of Peruvian Sheep. Preventive veterinary medicine 7, 225-228.
- Reiter-Owona, I., Petersen, E., Joynton, D., Aspöck, H., Darde, M.L., Disko, R., Dreazen, O., Dumon, H., Grillo, R., Gross, U., Hayde, M., Holliman, R., Ho-Yen, D.O., Janitschke, K., Jenum, P.A., Naser, K., Olszewski, M., Thulliez, P., Seitz, H.M., 1999. The past and present role of the Sabin-Feldman dye test in the serodiagnosis of toxoplasmosis. Bull World Health Organ 77, 929-935.
- Riyadh, R., 2005. Flock level sero-prevalence of and risk factors for *Toxoplasma gondii* in sheep and goats in northern Jordan. unpublished Master's thesis, Jordan University of Science and Technology, Irbid, Jordan.
- Robert-Gangneux, F., 2014. It is not only the cat that did it: how to prevent and treat congenital toxoplasmosis. J Infect 68 Suppl 1, S125-133.
- Rodger, S.M., Maley, S.W., Wright, S.E., Mackellar, A., Wesley, F., Sales, J., Buxton, D., 2006. Role of endogenous transplacental transmission in toxoplasmosis in sheep. Vet Rec 159, 768-772.
- Rossi, C.L., 1998. A simple, rapid enzyme-linked immunosorbent assay for evaluating immunoglobulin G antibody avidity in toxoplasmosis. Diagn Microbiol Infect Dis 30, 25-30.
- Rothe, J., McDonald, P.J., Johnson, A.M., 1985. Detection of *Toxoplasma* cysts and oocysts in an urban environment in a developed country. Pathology 17, 497-499.
- Rozette, L., Dumètre, A., Couquet, C.Y., Dardé, M.L., 2005. Seroprevalence de la toxoplasmose chez des ovins et des bovins en Haute-Vienne. Epidémiologie et Santé Animale 48, 97-99.
- Saavedra, R., Becerril, M.A., Dubeaux, C., Lippens, R., De Vos, M.J., Herion, P., Bollen, A., 1996. Epitopes recognized by human T lymphocytes in the ROP2 protein antigen of *Toxoplasma gondii*. Infect Immun 64, 3858-3862.
- Sabin, A.B., Feldman, H.A., 1948. Dyes as Microchemical Indicators of a New Immunity Phenomenon Affecting a Protozoon Parasite (*Toxoplasma*). Science 108, 660-663.
- Sager, H., Gloor, M., Björkman, C., Kritznier, S., Gottstein, B., 2003a. Assessment of antibody avidity in aborting cattle by a somatic *Neospora caninum* tachyzoite antigen IgG avidity ELISA. Vet Parasitol 112, 1-10.
- Sager, H., Gloor, M., Tenter, A., Maley, S., Hassig, M., Gottstein, B., 2003b. Immunodiagnosis of primary *Toxoplasma gondii* infection in sheep by the use of a P30 IgG avidity ELISA. Parasitol Res 91, 171-174.
- Sakata, F.B., Bellato, V., Sartor, A.A., de Moura, A.B., de Souza, A.P., Farias, J.A., 2012. *Toxoplasma gondii* antibodies sheep in Lages, Santa Catarina, Brazil, and comparison using IFA and ELISA. Revista brasileira de parasitologia veterinária = Brazilian journal of veterinary parasitology: Orgao Oficial do Colegio Brasileiro de Parasitologia Veterinaria 21, 196-200.

- Sedlák, K., Bártová, E., 2007. *Toxoplazmoza zvirat a její laboratorní diagnostika v České republice. Veterinarství* 57, 562-567.
- Seineke, P., 1996. Seroprävalenz von Antikörpern gegen *Toxoplasma gondii* bei Schafen, Zeigen. Schweinen in Niedersachsen. Dr. Med. Vet. Thesis, Hannover 171.
- Shaapan, R.M., El-Nawawi, F.A., Tawfik, M.A.A., 2008. Sensitivity and specificity of various serological tests for the detection of *Toxoplasma gondii* infection in naturally infected sheep. *Vet. Parasitol.* 153, 359-362.
- Shahiduzzaman, M., Islam, R., Khatun, M.M., Batanova, T.A., Kitoh, K., Takashima, Y., 2011. *Toxoplasma gondii* seroprevalence in domestic animals and humans in Mymensingh District, Bangladesh. *The Journal of veterinary medical science / the Japanese Society of Veterinary Science* 73, 1375-1376.
- Sharif, M., Gholami, S., Ziaei, H., Daryani, A., Laktarashi, B., Ziapour, S.P., Rafiei, A., Vahedi, M., 2007. Seroprevalence of *Toxoplasma gondii* in cattle, sheep and goats slaughtered for food in Mazandaran province, Iran, during 2005. *Vet J* 174, 422-424.
- Sharma, S., Sandhu, K.S., Bal, M.S., Kumar, H., Verma, S., Dubey, J.P., 2008. Serological survey of antibodies to *Toxoplasma gondii* in sheep, cattle, and buffaloes in Punjab, India. *J. Parasitol.* 94, 1174-1175.
- Sibley, L.D., Niesman, I.R., Parmley, S.F., Cesbron-Delauw, M.F., 1995. Regulated secretion of multi-lamellar vesicles leads to formation of a tubulo-vesicular network in host-cell vacuoles occupied by *Toxoplasma gondii*. *J Cell Sci* 108 ( Pt 4), 1669-1677.
- Siebert, P.D., Larrick, J.W., 1993. PCR MIMICS: competitive DNA fragments for use as internal standards in quantitative PCR. *Biotechniques* 14, 244-249.
- Sims, T.A., Hay, J., Talbot, I.C., 1988. Host-parasite relationship in the brains of mice with congenital toxoplasmosis. *J Pathol* 156, 255-261.
- Skjerve, E., Waldeland, H., Nesbakken, T., Kapperud, G., 1998. Risk factors for the presence of antibodies to *Toxoplasma gondii* in Norwegian slaughter lambs. *Preventive veterinary medicine* 35, 219-227.
- Splendore, A., 1908. Un nuovo protozoa parassita deconigli incontrato nelle lesioni anatomiche d'una malattia che ricorda in molti punti il Kala-azar dell'uomo. Nota preliminare pel. *Rev Soc Sci Sao Paulo* 3, 109-112.
- Standardization, W.E.C.o.B., 1968. Twentieth Report. Geneva, World Health Organization. (WHO Technical Report Series, No.384).
- Standardization, W.E.C.o.B., 1981. Thirty-first Report. Geneva, World Health Organization. (WHO Technical Report Series, No. 658).
- Steuber, S., Niu, A., Bauer, C., Reetz, J., Roth, A., Janitschke, K., 1995. The detection of *Toxoplasma gondii* in abortion tissues of sheep using the polymerase chain reaction. *Dtsch Tierarztl Wochenschr* 102, 91-93.

- Stimbirys, A., Bagdonas, J., Gerulis, G., Russo, P., 2007. A serological study on the prevalence of *Toxoplasma gondii* in sheep of Lithuania. *Pol J Vet Sci* 10, 83-87.
- Suzuki, Y., Conley, F.K., Remington, J.S., 1989. Importance of endogenous IFN-gamma for prevention of toxoplasmic encephalitis in mice. *J Immunol* 143, 2045-2050.
- Suzuki, Y., Orellana, M.A., Schreiber, R.D., Remington, J.S., 1988. Interferon-gamma: the major mediator of resistance against *Toxoplasma gondii*. *Science* 240, 516-518.
- Szeredi, L., Bacsadi, A., 2002. Detection of *Chlamydophila (Chlamydia) abortus* and *Toxoplasma gondii* in smears from cases of ovine and caprine abortion by the streptavidin-biotin method. *J Comp Pathol* 127, 257-263.
- Trees, A.J., Crozier, S.J., Buxton, D., Blewett, D.A., 1989. Serodiagnosis of ovine toxoplasmosis: an assessment of the latex agglutination test and the value of IgM specific titres after experimental oocyst-induced infections. *Res Vet Sci* 46, 67-72.
- Tumurjav, B., Terkawi, M.A., Zhang, H., Zhang, G., Jia, H., Goo, Y.K., Yamagishi, J., Nishikawa, Y., Igarashi, I., Sugimoto, C., Xuan, X., 2010. Serodiagnosis of ovine toxoplasmosis in Mongolia by an enzyme-linked immunosorbent assay with recombinant *Toxoplasma gondii* matrix antigen 1. *The Japanese journal of veterinary research* 58, 111-119.
- Tzanidakis, N., Maksimov, P., Conraths, F.J., Kiossis, E., Brozos, C., Sotiraki, S., Schares, G., 2012. *Toxoplasma gondii* in sheep and goats: seroprevalence and potential risk factors under dairy husbandry practices. *Vet Parasitol* 190, 340-348.
- van de Ven, E., Melchers, W., Galama, J., Camps, W., Meuwissen, J., 1991. Identification of *Toxoplasma gondii* infections by BI gene amplification. *J Clin Microbiol* 29, 2120-2124.
- van der Puije, W.N.A., Bosompem, K.M., Canacoo, E.A., Wastling, J.M., Akanmori, B.D., 2000. The prevalence of anti-*Toxoplasma gondii* antibodies in Ghanaian sheep and goats. *Acta tropica* 76, 21-26.
- Velge-Roussel, F., Marcelo, P., Lepage, A.C., Buzoni-Gatel, D., Bout, D.T., 2000. Intranasal immunization with *Toxoplasma gondii* SAG1 induces protective cells into both NALT and GALT compartments. *Infect Immun* 68, 969-972.
- Vesco, G., Buffolano, W., La Chiusa, S., Mancuso, G., Caracappa, S., Chianca, A., Villari, S., Currò, V., Liga, F., Petersen, E., 2007. *Toxoplasma gondii* infections in sheep in Sicily, southern Italy. *Vet. Parasitol.* 146, 3-8.
- Waldeland, H., 1977. Toxoplasmosis in sheep. Influence of various factors on the antibody contents. *Acta veterinaria Scandinavica* 18, 237-247.
- Waltner-Toews, D., Mondesire, R., Menzies, P., 1991. The seroprevalence of *Toxoplasma gondii* in Ontario sheep flocks. *Can Vet J* 32, 734-737.
- Wastling, J.M., Harkins, D., Buxton, D., 1994. Western blot analysis of the IgG response of sheep vaccinated with S48 *Toxoplasma gondii* (Toxovax). *Res Vet Sci* 57, 384-386.

- Wastling, J.M., Harkins, D., Maley, S., Innes, E., Panton, W., Thomson, K., Buxton, D., 1995. Kinetics of the local and systemic antibody response to primary and secondary infection with S48 *Toxoplasma gondii* in sheep. *J Comp Pathol* 112, 53-62.
- Wastling, J.M., Nicoll, S., Buxton, D., 1993. Comparison of two gene amplification methods for the detection of *Toxoplasma gondii* in experimentally infected sheep. *J Med Microbiol* 38, 360-365.
- Weissmann, J., 2003. Presumptive *Toxoplasma gondii* abortion in a sheep. *Can Vet J* 44, 322-324.
- Wilkins, M.F., O'Connell, E., Te Punga, W.A., 1988. Toxoplasmosis in sheep III. Further evaluation of the ability of a live *Toxoplasma gondii* vaccine to prevent lamb losses and reduce congenital infection following experimental oral challenge. *N Z Vet J* 36, 86-89.
- Williams, R.H., Morley, E.K., Hughes, J.M., Duncanson, P., Terry, R.S., Smith, J.E., Hide, G., 2005. High levels of congenital transmission of *Toxoplasma gondii* in longitudinal and cross-sectional studies on sheep farms provides evidence of vertical transmission in ovine hosts. *Parasitology* 130, 301-307.
- Wu, S.M., Danba, C., Huang, S.Y., Zhang, D.L., Chen, J., Gong, G., Xu, M.J., Yuan, Z.G., Zhu, X.Q., 2011. Seroprevalence of *Toxoplasma gondii* infection in Tibetan sheep in Tibet, China. *J Parasitol* 97, 1188-1189.
- Yai, L.E.O., Vianna, M.C.B., Soares, R.M., Cortez, A., Freire, R.L., Richtzenhain, L.J., Gennari, S.M., 2003. Evaluation of experimental *Toxoplasma gondii* (Nicolle and Manceaux, 1909) infection in pigs by bioassay in mice and polymerase chain reaction. *Brazilian Journal of Veterinary Research and Animal Science* 40, 227-234.
- Yang, N., Li, H., He, J., Mu, M., Yang, S., 2013. Seroprevalence of *Toxoplasma gondii* infection in domestic sheep in Liaoning Province, northeastern China. *J Parasitol* 99, 174-175.
- Yap, G.S., Sher, A., 1999. Cell-mediated immunity to *Toxoplasma gondii*: initiation, regulation and effector function. *Immunobiology* 201, 240-247.
- Zia-Ali, N., Fazaeli, A., Khoramizadeh, M., Ajzenberg, D., Darde, M., Keshavarz-Valian, H., 2007. Isolation and molecular characterization of *Toxoplasma gondii* strains from different hosts in Iran. *Parasitology Research* 101, 111-115.



## **PART II: AIMS OF THE STUDY**



In 2008, toxoplasmosis was identified as the fifth most important food-borne zoonotic pathogen in Belgium after *Salmonella* spp., *Campylobacter* spp., *Listeria monocytogenes* and verocytotoxigenic *Escherichia coli* (FAVV, 2008). Epidemiological data and case-control reports identify sheep as an important source of human toxoplasmosis. The literature review highlighted a gap in quantitative data on *T. gondii* prevalence in sheep in Belgium. The European Food Safety Authority has recommended the EU member states to implement control strategies for toxoplasmosis in animals and food products for human consumption, to reduce better the disease risk of *T. gondii*.

Aside from the gap in seroprevalence, significant gaps still exist in the pathogenesis and immune response during acute toxoplasmosis. Several experimental infections have been carried out, especially in mice, to investigate the host-immune responses during *T. gondii* infection. However, to our knowledge the distribution of the parasite and the correlated antibody and interferon (IFN)-gamma responses during the acute stage have not been investigated yet in larger animals. We studied the progress of a *T. gondii* infection in sheep with a special focus on the antibody and IFN-gamma responses as well as on the distribution of the parasite during the first three weeks after infection. Furthermore, the evolution of the immune response until two months after infection was studied. Since we observed strong antibody responses against several parasite antigens and clear T helper 1 cytokine responses in sheep, we were interested to see if a comparable immune response occurs in pigs.

The specific aims of this work are as follows:

- 1) To generate baseline data on the prevalence of ovine toxoplasmosis in Belgium
- 2) To study the acute *T. gondii* infection in sheep.

Following questions were addressed in this study:

- 2.1 Which small intestinal tissues are infected by the parasite in this acute phase and how long does the parasite reside in the gut?
- 2.2 Which immune response is evolving during this early phase? Here we specially focused on the IFN-gamma response, which is important for controlling the infection.
- 3) To study on a longer term - the first two months after infection - the humoral and cellular immune responses (Th1 versus Th2) in sheep against the parasite and to observe whether there is a relation between immune response and parasite distribution.
- 4) To compare the immune response and parasite distribution following a *T. gondii* infection in pigs with the results we obtained in sheep. Here, the pig model was chosen because pork is

the most commonly consumed meat in Belgium (and worldwide) (Worldwatch Institute) and because it was shown to be a risk factor for human toxoplasmosis in previous studies (Kijlstra and Jongert, 2009; Tenter et al., 2000)

## **PART III: EXPERIMENTAL STUDIES**



## **CHAPTER 3**

### ***SEROPREVALENCE OF *TOXOPLASMA GONDII* IN DOMESTIC SHEEP IN BELGIUM***

*Based on:* Verhelst, D., De Craeye, S., Vanrobaeys, M., Czaplicki, G., Dorny, P. and Cox, E.  
2014. Seroprevalence of *Toxoplasma gondii* in domestic sheep in Belgium. Vet Parasitol.  
205(1-2):57-61





### 3.1 Abstract

Although infected sheep are a potential source of *T. gondii* infection in humans, information is not available regarding the seroprevalence of *Toxoplasma gondii* in sheep in Belgium. We examined 3170 serum samples for anti-*Toxoplasma* IgG in sheep by a total lysate antigen (TLA) enzyme-linked immunosorbent assay (ELISA). The presence of IgG in sheep sera directed towards *T. gondii* could be demonstrated in 87.4 % of the tested sheep and in 96.2 % of the 209 tested flocks. The seroprevalences in Antwerp (65.2 %) and Wallonia (68.6 %) were significantly lower compared to the other regions in Belgium (96.7 % -97.8 %) ( $P < 0.05$ ). The present study is the first report that analyzed the prevalence of *T. gondii* infection in sheep in Belgium and confirms the high prevalence of *Toxoplasma*-specific IgG antibodies in the Belgian sheep population.

### 3.2 Introduction

Toxoplasmosis is a worldwide zoonosis caused by the obligatory protozoan *Toxoplasma gondii*. Almost all warm-blooded animals and humans can be infected (Yuan et al., 2013). Infections with *T. gondii* in sheep may lead to abortion and causes huge economic losses to the livestock industry (Tenter et al., 2000). In addition, consumption of non-frozen and undercooked sheep meat products are considered a source for human toxoplasmosis (Cook et al., 2000). The prevalence of *T. gondii* infection in sheep has been reported for many countries and ranged from 10 % in Slovakia (Kováčová, 1993) to 27.8 % in the Netherlands (Opsteegh et al., 2010), 65.6 % in France (Dumetre et al., 2006) and even 98% in Egypt (Ghoneim et al., 2010). The Belgian sheep population consists of 216,018 sheep divided over 27,253 flocks; 64.33 % of the sheep and 70.11% of the flocks are located in Flanders. Only 8,145 flocks are located in Wallonia, the French-speaking southern region of Belgium, corresponding to 55 % of the Belgian territory. The Flemish provinces East- and West-Flanders are the largest domestic sheep producers.

Considering the possible impact of *T. gondii* on human and veterinary health as well as the lack of knowledge regarding the prevalence in Belgium, the aim of this study is to determine the prevalence of *T. gondii* infection in Belgian sheep and sheep flocks.

### 3.3 Material and Methods

#### 3.3.1 Sample selection

Blood samples were collected from domestic sheep participating in the Visna-Maedi/CAE screening program of the Federal Agency for Safety of Food Chain (FASFC). These samples were from animals older than one year and from flocks from East-Flanders, West-Flanders, Antwerp, Limburg, Flemish Brabant and Wallonia. The distribution of samples over the sheep flocks of the different provinces and the number of sheep per provinces is presented in Table 3.1. After testing for Visna-Maedi/ Caprine Arthritis Encephalitis the 3170 serum samples were kept at -20°C until analysis for *T. gondii* antibodies.

**Table 3.1:** Seroprevalence of *T. gondii* in sheep and sheep flocks in different Flemish provinces and in Wallonia as determined by a TLA-specific antibody ELISA.

	Province	Flocks			Sheep			True Prevalence (95%CI)
		Total	N tested	N pos	Total	N tested	N pos (%)	
	Antwerp	2105	23	23	18326	402	240 (59.7%)	65.2% ( 58.5% - 62.6%)
	East-Flanders	7127	48	48	40508	563	479 (85.1%)	96.6% (92.1% - 99.6%)
Flandres	Flemish Brabant	2973	31	31	23144	370	323 (87.3%)	97.8% (93.8% - 99.8%)
	Limburg	1783	20	19	18459	256	222 (86.7%)	97.3% (92.5% - 99.7%)
	West Flanders	5120	36	36	38537	667	569 (85.3%)	96.8% (92.8% - 99.7%)
	Wallonia	8145	51	44	77044	912	567 (62.2%)	68.6% (63.0% - 74.8%)
Total		27253	209	201	216018	3170	2400 (75.7%)	87.4% (82.0% - 93.4%)

The blood samples were marked per flock but were not marked for specific characteristics such as age, breed, gender and farm management. The blood samples were tested for IgG antibodies to *T. gondii*, based on a *T. gondii*-specific antibody enzyme-linked immunosorbent assay (ELISA) following a protocol described further. One hundred samples were tested in indirect immunofluorescence assay (IFA) to confirm the results of the total lysate antigen (TLA) ELISA. The agreement between the results of the TLA ELISA and the IFA was assessed by calculating positive and negative agreement indices with credibility intervals according to the method described by Graham and Bull (1998) (Table 3.2). Considering the

results of those two tests, a, b, c and d are the observed results for each possible combination of ratings by the tests. a, being the number of positives with both tests, b, the number of samples testing negative in ELISA but positive in IFA, c, the number of samples testing positive in ELISA but negative in IFA, d, the number of negative samples in both tests. The proportion of specific agreement, positive agreement index (pr+) and negative agreement index (pr-), were calculated as follows:  $pr+ = 2a/(2a+b+c)$  and  $pr- = 2d/(2d+b+c)$ . The positive rating is an estimation of the conditional probability, given that one of the test results is positive, the other is also positive.

The true prevalences were estimated in a Bayesian analysis based on the model used by Speybroeck et al. (2013). The apparent antibody status of a sheep was linked to the true antibody status in terms of sensitivity (Se) and specificity (Sp) of the diagnostic test. Although we found a 100% agreement between the IFA and the TLA ELISA, these results were not retained as we could not validate the tests due to the lack of negative control samples. Therefore, we used the Se and Sp estimations from the previous study of Shaapan et al. (2008). The ELISA and the IFA had sensitivities of 90.1 % and 80.4%, respectively; and specificities of 85.9% and 91.4%, respectively. According to that study a Se range between 0.80 and 0.90 and a Sp range between 0.85 and 0.90 was used for the TLA ELISA. Credibility intervals (95%) of the true prevalences were calculated using the Bayesian model in Prevalence Package for R version 0.2.0 (Devleesschauwer et al., 2013; Speybroeck et al., 2013)

Statistical analysis of *T. gondii* prevalence between the different regions was performed using a Chi-square test using Prism (GraphPad). A *P*-value < 0.05 was considered statistically significant. Assuming a family-wise significance level of 0.05, individual *P*-values were considered significant if lower than Bonferroni corrected significance level of  $0.05/15=0.003$ .

### ***3.3.2 T. gondii-specific antibody enzyme-linked immunosorbent assay (ELISA)***

Serum samples were tested for antibodies against total lysate of *T. gondii* using a *T. gondii*-specific antibody enzyme-linked immunosorbent assay (ELISA). TLA was prepared as described by Scorza et al. (2003). Briefly, tachyzoites of the *T. gondii* RH strain, isolated from the peritoneal cavity of Swiss mice 4 days after intraperitoneal infection, were squeezed twice through a 26-Gauge needle and pelleted by centrifugation at 1000 x g. Next, the pellet was washed twice in PBS, followed by freezing, thawing and sonication on ice for 5 minutes

in an Ultrasonic disintegrator (MSE, Leicester, United Kingdom) to solubilize the *T. gondii* tachyzoite antigens. The ELISA was performed as described by Verhelst et al. (2011). Briefly, 96-well Nunc Maxisorp™ plates were coated overnight with TLA at a concentration of 10 µg ml<sup>-1</sup> bicarbonate coating buffer (pH 9.4) at 4°C. Afterwards the plate was blocked with PBS 5% non fat milk powder for 2 hours at 37°C, and incubated with the serum samples, being diluted 100 fold into PBS 0.2% Tween® 20, for 1 hour at 37°C. An anti-sheep IgG horseradish peroxidase conjugated rabbit antiserum (AbD Serotec, Belgium), appropriately diluted in PBS 0.2% Tween 20® was added for 60 min. at 37°C. In between each step, plates were washed 5 times with PBS 0.01% Tween20®. As a last step, a 3,3',5,5'-tetramethylbenzidine buffer was added for 30 min. at room temperature, whereafter the OD at 450 nm was determined with an ELISA reader. Positive and negative control serum samples at the same dilution of the serum samples were included on each plate. A corrected optical density (OD) was calculated as the OD of a sample – OD of the negative control samples.

### **3.3.3 Indirect immunofluorescence antibody test (IFA)**

One hundred samples were tested in IFA to confirm the results of the ELISA. Fifty microliters of the 1/50 diluted serum in PBS was applied for 30 min. at 37°C on a slide coated with formalin fixed tachyzoites from the RH strain (Toxo-Spot IF, Biomérieux, Marcy-l'Etoile, France). In subsequent steps, the slides were washed with PBS, incubated for 30 min. at 37°C with 30 µl of a 1/25 in PBS-Evans Blue diluted fluorescein isothio-cyanate (FITC) conjugated rabbit anti-sheep IgG (KPL, Maryland, USA), washed again, dried and examined by fluorescence microscopy.

## **3.4 Results and Discussion**

A total of 3170 ovine serum samples were tested by TLA-ELISA for the presence of anti-*T. gondii* IgG. When comparing the one hundred samples tested in IFA and TLA ELISA, positive and negative agreement indices with their 95 % credibility intervals (between brackets) were used to assess the agreement between both tests (Table 3.2). A positive agreement of 1 (0.94-1.00) and a negative agreement of 1 (0.91-1.00) were determined. The literature mentioned estimates of sensitivity between 90.1 % and 97.8 % and of specificity between 85.9 % and 96.4 % for the TLA ELISA (Opsteegh et al., 2010; Shaapan et al., 2008).

**Table 3.2:** Results of the serological tests on sheep sera. Comparison between the TLA-specific antibody ELISA and the IFA.Positive agreement index:  $pr+ = 2a/(2a+b+c) = 2*63/(2*63+0+0)$ Negative agreement index  $pr- = 2d/(2d+b+c) = 2*37/(2*37+0+0)$ 

	IIFA	
	+	-
TLA ELISA		
+	63 (a)	0 (c)
-	0 (b)	37 (d)

The 3170 ovine serum samples, obtained from “Animal Health Care Flanders and Wallonia (DGZ and ARSIA), were sampled as part of the Visna-Maedi/ CAE screening program of the Federal Agency for Safety of Food Chain (FASFC). As mentioned above, no information was available about the farms, flock size, age, gender or breed. Table 3.1 gives an overview of the sheep population in Belgium, and shows the results of the tested sheep flocks and the seroprevalence per province. We found a very high seroprevalence of 87.4 % (CI 0.81-0.93) in the Belgian sheep population. This is much higher than in the Netherlands (27.8 %) (Opsteegh et al., 2010) and higher than in other European countries such as France, with a seroprevalence of 65.6 % (Dumetre et al., 2006) or Great-Britain with 74 % (Hutchinson et al., 2011).

To evaluate whether the seroprevalence in Belgian sheep matches a geographical distribution, the results of our study were grouped per region. Small regional differences were observed and are shown in Table 3.1. The province of Antwerp showed the lowest seroprevalence (65.2 %), followed by the Walloon region (68.6 %), East Flanders (96.7 %), West Flanders (96.8 %) and Limburg (97.3 %). Flemish Brabant noted the highest seroprevalence namely, 97.8%. The *T. gondii* seroprevalence in Antwerp and Wallonia were significantly lower than those in the other regions ( $P < 0.0001$ ) (Table 3.3). Only 8 of the 209 tested flocks were seronegative; among these, one flock was located in Flanders, more specifically in Limburg, the other 7 seronegative flocks were located in Wallonia.

**Table 3.3:** *P*-value between the provinces

<b>P-value</b>	Antwerp	East-Flanders	Flemish Brabant	Limburg	West Flandres	Wallonia
Antwerp	-	<0.0001	<0.0001	<0.0001	<0.0001	0.698
East-Flanders	-	-	0.635	0.826	0.993	<0.0001
Flemish Brabant	-	-	-	0.978	0.676	<0.0001
Limburg	-	-	-	-	0.86	<0.0001
West Flandres	-	-	-	-	-	<0.0001
Wallonia	-	-	-	-	-	-

These results show the widespread distribution of *T. gondii* infection in sheep flocks in Belgium.

Innes et al. (2009a) mentioned that regional differences in the level of soil contamination may lead to variation in the seroprevalence as sheep get *T. gondii* infections mainly from ingesting oocysts from the environment. In that respect, it is important to note that sporulated oocysts can remain viable and infective in the environment for several months, depending on temperature and relative humidity (Dubey, 1988; Innes et al., 2009a; Owen and Trees, 1999; Plant et al., 1974). Toxoplasmosis in sheep starts mostly by the ingestion of as few as 200 sporulated oocysts (McColgan et al., 1988). As sheep are not carnivores, the only two possible infection routes are: 1) the vertical from mother to foetus during pregnancy, that represents a serious risk for congenital disease (Dubey, 2009b), 2) the ingestion of oocysts during grazing. Congenital transmission of *T. gondii* is reported in several studies (Buxton et al., 2007; Innes et al., 2009b). An infection in the early gestation is mostly fatal due to the absence of a foetal immune response, whereas infection in the late pregnancy, after day 120 of gestation, most of the time results in the birth of normal lambs, which may be infected and immune (Blewett and Watson, 1983). In subsequent pregnancies the effective immune response developed by the infected animals, will protect them against disease (Innes et al., 2009b). Nevertheless, it has been shown that in sheep repeated transplacental transmission of *T. gondii* can occur (Morley et al., 2005; Morley et al., 2008). In Belgium, the obtained seroprevalence cannot be linked with abortion, since there are no official data.

Associations have been made between exposure of sheep to *T. gondii* and the presence of cats in farms or the circulation of stray cats (Plant et al., 1974). It has been noted that environmental oocyst contamination is concentrated in and around cat defecation sites

(Afonso et al., 2008). After a primary infection, cats continuously shed oocysts in their faeces for 4 to 14 days with a peak of tens of millions at 6-8 days (Dubey, 2009a; Dubey and Frenkel, 1972; Skjerve et al., 1998). There are only two reports on the seroprevalence of toxoplasmosis in Belgian cats, one in the stray cat population of Ghent (Dorny et al., 2002) and one in Belgian pet cats (De Craeye et al., 2008). In the stray cat population, a very high *T. gondii* seroprevalence of 70.2 % was found (243/346) (Dorny et al., 2002), but this was not the case when testing serum samples of pet cats throughout Belgium (De Craeye et al., 2008). The geographical differences in cat seroprevalence observed in the study of De Craeye et al (2008) do not correspond with those in sheep seroprevalence observed in our study. A possible reason is that the pet cat population studied by De Craeye et al (2008) does not contribute much to infection of pastures on which sheep graze.

Flock location has been reported as another reason for variation in seroprevalence in sheep (Caballero-Ortega et al., 2008; Halos et al., 2010; Skjerve et al., 1998). However, due to privacy regulations, the exact location of the flocks cannot be released. We assume that the difference is not due to housing conditions since Belgian sheep are kept either totally outdoor or indoor with outdoor access. The differences we found among the provinces should most likely be seen as a result from differences in environmental contamination, nevertheless we could not identify etiological factors.

In conclusion, this is the first report of *T. gondii* seroprevalence in sheep in Belgium, demonstrating a very high seroprevalence in sheep and flocks. As a consequence, this study supports the hypothesis that mutton is a major source of human toxoplasmosis.

## **Acknowledgements**

This work was funded by a PhD grant of the Institute for the Promotion of Innovation through Science and Technology in Flanders (IWT-Vlaanderen) and the Belgian Federal Public Service for Health, Food Chain Safety and Environment, grant RF6176

### 3.5 References

- Afonso, E., Lemoine, M., Poulle, M.L., Ravat, M.C., Romand, S., Thulliez, P., Villena, I., Aubert, D., Rabilloud, M., Riche, B., Gilot-Fromont, E., 2008. Spatial distribution of soil contamination by *Toxoplasma gondii* in relation to cat defecation behaviour in an urban area. *Int J Parasitol* 38, 1017-1023.
- Blewett, D.A., Watson, W.A., 1983. The epidemiology of ovine toxoplasmosis. II. Possible sources of infection in outbreaks of clinical disease. *Br Vet J* 139, 546-555.
- Buxton, D., Maley, S.W., Wright, S.E., Rodger, S., Bartley, P., Innes, E.A., 2007. Ovine toxoplasmosis: transmission, clinical outcome and control. *Parassitologia* 49, 219-221.
- Caballero-Ortega, H., Palma, J.M., Garcia-Marquez, L.J., Gildo-Cardenas, A., Correa, D., 2008. Frequency and risk factors for toxoplasmosis in ovines of various regions of the State of Colima, Mexico. *Parasitology* 135, 1385-1389.
- Cook, A.J., Gilbert, R.E., Buffolano, W., Zufferey, J., Petersen, E., Jenum, P.A., Foulon, W., Semprini, A.E., Dunn, D.T., 2000. Sources of *toxoplasma* infection in pregnant women: European multicentre case-control study. European Research Network on Congenital Toxoplasmosis. *BMJ* 321, 142-147.
- De Craeye, S., Francart, A., Chabauty, J., Van Gucht, S., Leroux, I., Jongert, E., 2008. Toxoplasmosis in Belgian pet cats: recommendations for owners. *Vlaams Diergeneeskundig Tijdschrift* 77, 325-330.
- Devleesschauwer, B., Torgerson, P., Charlier, J., Levecke, B., Praet, N., Dorny, P., Berkvens, D., Speybroeck, N., 2013. prevalence: Tools for prevalence assessment studies. R package version 0.2.0. <http://cran.r-project.org/package=prevalence>
- Dorny, P., Speybroeck, N., Verstraete, S., Baeke, M., De Becker, A., Berkvens, D., Vercruysse, J., 2002. Serological survey of *Toxoplasma gondii*, feline immunodeficiency virus and feline leukaemia virus in urban stray cats in Belgium. *Vet Rec* 151, 626-629.
- Dubey, J.P., 2009a. History of the discovery of the life cycle of *Toxoplasma gondii*. *Int J Parasitol* 39, 877-882.
- Dubey, J.P., 2009b. Toxoplasmosis in sheep--the last 20 years. *Vet Parasitol* 163, 1-14.
- Dubey, J.P., Frenkel, J.K., 1972. Cyst-induced toxoplasmosis in cats. *J Protozool* 19, 155-177.
- Dubey, J.P.a.B., C. P., 1988. Toxoplasmosis of animals and man. CRC Press, Inc., Boca Raton, FL 33431, USA, 220 p.
- Dumetre, A., Ajzenberg, D., Rozette, L., Mercier, A., Darde, M.L., 2006. *Toxoplasma gondii* infection in sheep from Haute-Vienne, France: seroprevalence and isolate genotyping by microsatellite analysis. *Vet Parasitol* 142, 376-379.



- Ghoneim, N.H., Shalaby, S.I., Hassanain, N.A., Zeedan, G.S., Soliman, Y.A., Abdalhamed, A.M., 2010. Comparative study between serological and molecular methods for diagnosis of toxoplasmosis in women and small ruminants in Egypt. *Foodborne pathogens and disease* 7, 17-22.
- Graham, P., Bull, B., 1998. Approximate standard errors and confidence intervals for indices of positive and negative agreement. *J Clin Epidemiol* 51, 763-771.
- Halos, L., Thebault, A., Aubert, D., Thomas, M., Perret, C., Geers, R., Alliot, A., Escotte-Binet, S., Ajzenberg, D., Darde, M.L., Durand, B., Boireau, P., Villena, I., 2010. An innovative survey underlining the significant level of contamination by *Toxoplasma gondii* of ovine meat consumed in France. *Int J Parasitol* 40, 193-200.
- Hutchinson, J.P., Wear, A.R., Lambton, S.L., Smith, R.P., Pritchard, G.C., 2011. Survey to determine the seroprevalence of *Toxoplasma gondii* infection in British sheep flocks. *Vet Rec* 169, 582.
- Innes, E.A., Bartley, P.M., Buxton, D., Katzer, F., 2009b. Ovine toxoplasmosis. *Parasitology* 136, 1887-1894.
- Innes, E.A., Bartley, P.M., Maley, S., Katzer, F., Buxton, D., 2009a. Veterinary vaccines against *Toxoplasma gondii*. *Mem Inst Oswaldo Cruz* 104, 246-251.
- Kováčová, D., 1993. Sérologická prevalence *Toxoplasma gondii* u oviec. *Veterinarstvi* 43, 51-54.
- McColgan, C., Buxton, D., Blewett, D.A., 1988. Titration of *Toxoplasma gondii* oocysts in non-pregnant sheep and the effects of subsequent challenge during pregnancy. *Vet Rec* 123, 467-470.
- Morley, E.K., Williams, R.H., Hughes, J.M., Terry, R.S., Duncanson, P., Smith, J.E., Hide, G., 2005. Significant familial differences in the frequency of abortion and *Toxoplasma gondii* infection within a flock of Charollais sheep. *Parasitology* 131, 181-185.
- Morley, E.K., Williams, R.H., Hughes, J.M., Thomasson, D., Terry, R.S., Duncanson, P., Smith, J.E., Hide, G., 2008. Evidence that primary infection of Charollais sheep with *Toxoplasma gondii* may not prevent foetal infection and abortion in subsequent lambings. *Parasitology* 135, 169-173.
- Opsteegh, M., Teunis, P., Mensink, M., Zuchner, L., Titilincu, A., Langelaar, M., van der Giessen, J., 2010. Evaluation of ELISA test characteristics and estimation of *Toxoplasma gondii* seroprevalence in Dutch sheep using mixture models. *Preventive veterinary medicine* 96, 232-240.
- Owen, M.R., Trees, A.J., 1999. Genotyping of *Toxoplasma gondii* associated with abortion in sheep. *J Parasitol* 85, 382-384.
- Plant, J.W., Richardson, N., Moyle, G.G., 1974. *Toxoplasma* infection and abortion in sheep associated with feeding of grain contaminated with cat faeces. *Aust Vet J* 50, 19-21.

- Scorza, T., D'Souza, S., Laloup, M., Dewit, J., De Braekeleer, J., Verschueren, H., Vercammen, M., Huygen, K., Jongert, E., 2003. A GRA1 DNA vaccine primes cytolytic CD8(+) T cells to control acute *Toxoplasma gondii* infection. *Infect Immun* 71, 309-316.
- Shaapan, R.M., El-Nawawi, F.A., Tawfik, M.A.A., 2008. Sensitivity and specificity of various serological tests for the detection of *Toxoplasma gondii* infection in naturally infected sheep. *Vet. Parasitol.* 153, 359-362.
- Skjerve, E., Waldeland, H., Nesbakken, T., Kapperud, G., 1998. Risk factors for the presence of antibodies to *Toxoplasma gondii* in Norwegian slaughter lambs. *Preventive veterinary medicine* 35, 219-227.
- Speybroeck, N., Devleeschauwer, B., Joseph, L., Berkvens, D., 2013. Misclassification errors in prevalence estimation: Bayesian handling with care. *International journal of public health* 58, 791-795.
- Tenter, A.M., Heckeroth, A.R., Weiss, L.M., 2000. *Toxoplasma gondii*: from animals to humans. *Int J Parasitol* 30, 1217-1258.
- Verhelst, D., De Craeye, S., Dorny, P., Melkebeek, V., Goddeeris, B., Cox, E., Jongert, E., 2011. IFN-gamma expression and infectivity of *Toxoplasma* infected tissues are associated with an antibody response against GRA7 in experimentally infected pigs. *Vet Parasitol* 179, 14-21.
- Yuan, Z.G., Luo, S.J., Dubey, J.P., Zhou, D.H., Zhu, Y.P., He, Y., He, X.H., Zhang, X.X., Zhu, X.Q., 2013. Serological evidence of *Toxoplasma gondii* infection in five species of bats in China. *Vector Borne Zoonotic Dis* 13, 422-424.

## **CHAPTER 4**

### ***PARASITE DISTRIBUTION AND ASSOCIATED IMMUNE RESPONSE DURING THE ACUTE PHASE OF *TOXOPLASMA GONDII* INFECTION IN SHEEP***

*Based on:* Verhelst, D., De Craeye, S., Entrican, G., Dorny, P. and Cox, E. Parasite distribution and associated immune response during the acute phase of a *Toxoplasma gondii* infection in sheep. Submitted



## 4.1 Abstract

In many countries, *Toxoplasma gondii* (*T. gondii*) is a major cause of reproductive disorders and abortions in the sheep industry, and therefore responsible for important financial and economic losses. In addition, consumption of undercooked infected lamb is an important risk factor for human toxoplasmosis.

In the present study, sheep were orally infected with *T. gondii* tissue cysts to investigate the initial phase of the infection: the parasites' entry site, the subsequent distribution of the parasite and the host-immune response. The presence of parasite DNA, the interferon-gamma (IFN-gamma) and interleukine-12 (IL-12) responses were determined at 4, 8, 10, 14 and 21 days post infection (dpi) in the small intestinal mucosa, the draining mesenteric lymph nodes (MLN), the popliteal LN, the spleen and blood leukocytes (PBMC). Furthermore, appearance of *Toxoplasma*-specific IgM and IgG in serum was tested daily by an indirect immunofluorescence assay and total IgG, IgG1, IgG2 and IgA by a total parasitic lysate-specific enzyme-linked immunosorbent assay. Parasite DNA already appeared in the cranial small intestinal mucosa the first days after infection. Simultaneously, high IFN-gamma and IL-12 responses were induced, mainly in the mesenteric LN. The emergence of IgG1 (at 8 dpi), and IgG2 (at 11 dpi) was accompanied by a decrease or even disappearance of the IFN-gamma and IL-12 response in the Peyer's patches (PP), PBMC and popliteal LN. Meanwhile, the parasite DNA could be recovered from most mucosal and systemic tissues while it became undetectable in the small intestine, popliteal LN, PBMC and spleen 21 dpi, suggesting that the parasite is leaving these tissues.

Our results indicate that parasites enter the cranial small intestine the first days after infection and that after an increase the first two weeks after infection, the parasite DNA levels in the intestine drop below the detection limit three weeks after infection. This coincides with an increase in parasitic-specific serum IgG1 and IgG2 responses and a decrease of the antigen-specific IFN-gamma response in PP, PBMC and popliteal LN. We suggest a role for IFN-gamma and IL-12 during acute infection.

## 4.2 Introduction

*Toxoplasma gondii* is an obligate intracellular parasite with a worldwide prevalence in a wide variety of hosts (Cenci-Goga et al., 2011). In many countries, the parasite is a major cause of reproductive disorders, miscarriages and abortions in the sheep industry, and therefore responsible for important financial and economic losses (Buxton et al., 2007b; EFSA, 2007). Based on data collected by the Veterinary Investigation Diagnosis Analysis in 2009 (VIDA, 2009), toxoplasmosis is the second diagnosed cause of abortion in sheep in the UK (25.4 %), after *Chlamydia abortus* (45.6 %).

Undercooked infected lamb is considered an important risk factor for human toxoplasmosis (Cook et al., 2000). Infection in herbivorous animals occurs through ingestion of *T. gondii* oocysts excreted by cats. One week after a first infection, cats can shed over 100 million oocysts in their faeces during a period of 7-14 days depending on the infection stage and the immune status of the cat. Sporulated oocysts can stay infectious in the environment during several months and contaminate pastures, feeds and also drinking water (Dubey, 1988; Innes et al., 2009b).

An acute primary infection during gestation in the ewe will result in the transmission of the parasite to the foetus. As in humans, the consequences for the foetus depend on the stage of the gestation: an early infection (during the first or second trimester of gestation) will result in fetal death and resorption or abortion, whereas a later infection will lead to the birth of clinically normal but latently infected lambs or lambs with symptoms of congenital toxoplasmosis (fever, growth retardation, weakness) (Esteban-Redondo and Innes, 1997; Pappas et al., 2009; Rodger et al., 2006).

The immunity developed by the pregnant ewe will protect it against infection of the foeti during subsequent gestations (Innes et al., 2009b). The same protection develops when a non-pregnant ewe becomes infected with *T. gondii* (Buxton, 1998; Rodger et al., 2006). Nevertheless, data of Morley et al. (2005, 2008) suggested that immunity might protect less well against transplacental transmission than thought. These findings were contested by several authors, as these were based on *T. gondii* DNA detection only and not on the demonstration of *T. gondii*-associated lesions by histopathology. In addition, the authors didn't exclude other abortifacient agents (Dubey, 2009b). Williams et al. (2005) suggested that the observations of Morley et al. (2005, 2008) were due to breed differences.

Whereas several experimental infections have been carried out in sheep to study host-immune responses, to our knowledge, the distribution of the parasite and the correlated

antibody and interferon (IFN)-gamma responses during acute ovine toxoplasmosis after oral infection have not been studied yet (Blewett et al., 1982; Blewett and Watson, 1983; Buxton et al., 2007a, b; Dubey, 2009b; Innes et al., 2009b)

In the present study, oral infections of sheep, with *T. gondii* tissue cysts, were performed to determine the entry site and subsequent distribution of the parasite following the oral infection route. Furthermore, we monitored the local IFN-gamma response during the acute phase of the infection, because of its important role as a mediator of host resistance against *Toxoplasma* as demonstrated in mice, sheep and humans (Hunter et al., 1994; Innes et al., 1995b; Torres-Morales et al., 2014).

### **4.3 Material and Methods**

#### ***4.3.1 Animals and experimental procedure***

Thirteen 7-week-old conventionally reared lambs were selected (Belgian cross-breed, Zootechnical Centre, Leuven, Belgium), based on their *T. gondii* and *Neospora caninum* seronegative status. The absence of antibodies against *T. gondii* was assessed by an indirect immunofluorescence assay (IFA, Toxo-Spot IF, Biomérieux, Marcy-l'Etoile, France), the absence of antibodies against *Neospora* with the *Neospora caninum* Antibody Test Kit (CHEKIT\* Neospora, Idexx, Hoofddorp, The Netherlands). The latter were tested since *Neospora* antibodies can cross-react with *T. gondii*.

After an acclimation period of one week at the experimental animal facilities of Ghent University, eleven animals were orally infected with 3000 tissue cysts of the *T. gondii* Prugniaud strain (PRU). PRU is a type II genotype isolated from a case of human lethal congenital toxoplasmosis (Bivas-Benita et al., 2003; Martrou et al., 1965). Sheep were killed by captive bolt and exsanguination on day 4 (4 dpi; N=3), 8 (8 dpi; N=2), 10 (10 dpi; N=2), 14 (14 dpi; N=1) and 21 (21 dpi; N=3) after infection. Two animals were kept as negative controls (C) and euthanized at 21 days. Blood samples were taken daily from the jugular vein of each animal, until euthanasia, for monitoring the *T. gondii*-specific serum antibody response. At euthanasia, portions of duodenal (D), jejunal (J) and ileal (I) tissue with and without Peyer's patches, the draining mesenteric lymph nodes (MLN), popliteal LN, spleen and blood on heparin were sampled. Samples were processed as described further, to

determine the spread of the parasite as well as the appearance of a specific IFN-gamma response in the different tissues at the early phase of the infection.

All experimental and animal management procedures were approved by the Animal Care and Ethics Committee of the faculties of Bioscience Engineering and Veterinary Medicine, Ghent University (2007/103).

#### **4.3.2 Inoculum**

Tissue cysts of the *T. gondii* PRU strain were harvested from the brains of chronically infected C57BL6 mice and the number of brain cysts was calculated as described by Verhelst et al. (2011). Briefly, infected mice were euthanized five weeks after infection and their brains were homogenized in phosphate buffered saline (PBS) using a Potter Homogenizer. The concentration of brain cysts in the suspension was determined by phase contrast microscopy whereafter the brain homogenate was diluted in PBS to obtain a final concentration of 300 cysts per ml. The sham inoculate given to the control animals was prepared similarly from non-infected mouse brains.

#### **4.3.3 Purification of recombinant antigens**

The recombinant antigens rGRA7, rEC2, rMIC3 were used to stimulate cell suspensions for IFN-gamma and IL-12 production. Recombinant GRA7 has already been demonstrated to be useful as a sero-diagnostic marker of acute and chronic toxoplasmosis (Aubert et al., 2000; Bonhomme et al., 1998; Jacobs et al., 1999). Furthermore GRA7 is expressed by all infectious stages of *T. gondii* and appears plentiful on the surface of the host cells, the host cell cytosol, the parasitophorous vacuolar membrane and lumen (Holec-Gasior, 2013). After rupture of the host cells, high amounts of GRA7 come in contact with the host immune system, triggering strong antibody responses (Fischer et al., 1998). The recombinant rEC2 antigen is a chimera of the antigenic regions of the microneme proteins MIC2 and MIC3 and of the surface antigen SAG1.

Briefly, the HIS-tagged recombinant antigens were purified from an *E. coli* culture using metal chelate affinity chromatography (Ni-NTA Superflow, Qiagen, GmbH, Hilden, Germany), according to the manufacturer's instructions. The recombinant antigens were eluted from the Ni-NTA columns by incubating in 500 mM imidazol buffer followed by



dialysis through a CelluSep H1 membrane (Membrane Filtration Products, Texas, USA) in urea buffer for rEC2, rGRA7 and rROP2 or PBS for rMIC3.

*Toxoplasma* total lysate antigen (TLA) was produced as described before (Vercammen et al., 2000). Briefly, *T. gondii* RH tachyzoites (type I strain) were harvested from the peritoneal cavity of Swiss mice that were intraperitoneally infected 4 days earlier. The ascites fluid was passed twice through a 26-gauge needle. After centrifugation for 15 min at 950 x g, the pellet, containing the tachyzoites, was washed twice in PBS, followed by sonication in an Ultrasonic disintegrator (MSE, Leicester, United Kingdom) to solubilize the *T. gondii* tachyzoite antigens. The total protein concentration was measured by the bicinchoninic acid (BCA) reaction (Thermo Scientific Pierce BCA Protein Assay Kit, Erembodegem, Belgium), whereafter the purified antigens were aliquoted and stored at -20 °C until use.

#### **4.3.4 Indirect Immunofluorescence Antibody Test (IFA)**

To test the presence of specific IgG and IgM antibodies against *T. gondii*; the sheep sera were diluted 1:50 in PBS and tested as follows: fifty microliter of this dilution was applied on a slide coated with formalin-treated *T. gondii* RH tachyzoites (Toxo-Spot IF, Biomérieux, Marcy-l'Etoile, France) for 30 min at 37 °C. On each slide, seronegative and seropositive sheep reference serum samples were used as controls. After washing with PBS, 30 µl of a 1:25 in PBS-Evans blue diluted fluorescein isothiocyanate conjugated rabbit anti-sheep IgM or rabbit anti-sheep IgG (KPL, Maryland, USA) was applied for 30 min at 37°C. The slides were then washed again; air dried and examined using fluorescence microscopy.

#### **4.3.5 *T. gondii* -specific antibody ELISA**

The presence of *T. gondii* antigen-specific IgG, IgG1, IgG2 and IgA in the collected sera was tested by ELISA. Nunc maxisorp immunoplates (Life Technologies, Merelbeke, Belgium) were coated overnight at 4°C with TLA, recombinant GRA7 or rROP2 at a concentration of 10 µg/ml in bicarbonate coating buffer (pH 9.7). In subsequent steps, the plates were blocked, during 2 h at 37°C, in 1% non-fat milk powder in PBS; incubated for 1 h at 37°C with 100-fold diluted sera in PBS with 0.05% Tween<sup>®</sup> 20 and 1% non-fat milk powder. This was followed by an incubation step, for 1 h at 37°C, with rabbit anti-sheep IgG- or IgA-labeled with horseradish peroxidase (AbD Serotec (Gentaur), Brussels, Belgium) or with a mouse anti-bovine IgG1 or IgG2 secondary antibody followed by an incubation with

an anti-mouse immunoglobulin-horseradish peroxidase conjugate. Subsequently a 3,3',5,5'-tetramethylbenzidine solution (TMB)(Sigma-Aldrich, Diegem, Belgium) was added. In between each step, the plates were manually washed 5 times with PBS 0.05% Tween<sup>®</sup> 20. The reaction was stopped by adding 50 µl 2 M H<sub>2</sub>SO<sub>4</sub> (stop solution). Absorbance was read at 450 nm. Positive and negative control sera were included on each plate. The corrected optical density (OD) was calculated as: OD of sample - OD of negative control sample. The serum samples were considered positive when the corrected optical density (OD<sub>450</sub>) of the dilutions exceeded the cut-off value (= mean OD<sub>450</sub> (negative controls) + 3 x its standard deviation). The negative control sera in the IFA and ELISA were from *T. gondii* negative sheep. They tested negative in the Modified agglutination test, the IFA and the Sabin Feldman Dye Test.

#### ***4.3.6 Isolation of mononuclear cells (MC) from blood, spleen, lymph nodes and intestine***

The MC from blood and spleen were isolated as described by Verhelst et al. (2001). The peripheral blood MCs (PBMCs) were isolated by density gradient centrifugation using Lymphoprep<sup>™</sup> (Nycomed, Brussels, Belgium). Briefly, heparinized blood was centrifuged during 25 min at 1000 x g and 18°C. After removing the plasma layer, the buffy coat and erythrocyte pellet were suspended in an equal volume of PBS with 1mM ethylenediaminetetraacetic acid (PBS-EDTA) and layered onto the gradient. Tubes were centrifuged for 45 min at 800 x g and 18°C. The interface layer, containing the MCs, was collected and the cells were washed in PBS-EDTA.

Splenocytes were isolated from the spleen after removing the surrounding fat. The obtained cell suspension was further purified by lysing the erythrocytes in the presence of ammonium chloride. After centrifugation (380 x g at 18°C for 10 min), the pelleted cells were washed and resuspended in PBS-EDTA (1mM).

The MCs from MLN, popliteal LN, duodenal, jejunal and ilial Peyer's patches (PP) were isolated as described by Van den Broeck et al. (1998) and the MCs from the duodenal, jejunal and ileal lamina propria as described by Vande Walle et al. (2011). All isolated cells were resuspended at a final concentration of  $1 \times 10^7$  cells/ml in complete leucocyte medium (RPMI 1640, Gibco, Merelbeke, Belgium) supplemented with 10% fetal calf serum (FCS, Greiner Bio-One, Belgium), 200 mM L-glutamin (Gibco), 100 U/ml penicillin and 100 µg/ml streptomycin (P/S; Gibco), 100 mM non-essential amino acids (Gibco) and 100 µg/ml kanamycin (Gibco).

#### ***4.3.7 Isolation of enterocytes from the intestine***

At euthanasia, duodenal, jejunal and ileal segments without PP of approximately one meter were sampled, flushed three times with Krebs buffer (0.12 M NaCl, 0.014 M KCl, 0.001 M KH<sub>2</sub>PO<sub>4</sub>, 0.025 M NaHCO<sub>3</sub>, pH 7.4) at room temperature and filled for 95% with Hank's Buffered Salt Solution (HBSS) supplemented with 1.5mM EDTA + 1mM 1,4-dithiothreitol (DTT) and pre-heated at 37 °C. Then the segments were incubated in 2 liter PBS for 15 min at 37°C. Thereafter the contents were collected and placed at 4°C. This process was repeated twice. The isolated cells were pooled and washed three times with HBSS supplemented with 0.1mM phenylmethylsulfonyl fluoride (PMSF). PMSF is a serine protease inhibitor that prevents proteolytic degradation of proteins (James, 1978). After centrifugation for 10 min at 1800 x g and 4°C, the cell pellets were resuspended at a final concentration of 10<sup>7</sup> cells/ml in complete medium.

#### ***4.3.8 Detection of parasites in cell populations by real-time PCR***

DNA was extracted from cell suspensions using the Qiagen QIAmp DNA Mini kit (Qiagen GmbH, Hilden, Germany), according to the manufacturer's instructions. After that, the presence of *T. gondii* DNA was determined by duplex qPCR analysis as described before (Kijlstra et al., 2008): the *T. gondii* repeat element (AF146527) was used as target for the detection of the parasite's DNA and the amplification of the cellular 18S rRNA gene. Since host cells are always present in the sample, the latter reaction should always be positive. Tenfold dilutions of a counted number of RH tachyzoites and cultured swine kidney cells (SK-6) were used to generate a standard line for the quantification of *T. gondii* parasites and one for the cells, respectively. Real-time qPCR was performed with an initial 3 min denaturation step at 95 °C, followed by 45 cycles at 95°C for 15 s and at 60°C for 20 s.

#### ***4.3.9 Restimulation of isolated mononuclear cells with T. gondii antigens***

The MCs were seeded in 96-well flat bottom cell culture plates (Greiner bio-one) at 10<sup>6</sup> cells per 100 µl complete medium and per well. After an initial incubation of 12 h, the mitogen concanavalin A (Con A) (5 µg/ml) (BD Biosciences) or 10 µg/ml of one of the following antigens was added: rEC2, rGRA7, rMIC3 or, total lysate antigen (TLA). The recombinant proteins were diluted in complete medium, supplemented with 10 µg/ml

polymyxin B to neutralize potentially contaminating endotoxin (Cardoso et al., 2007). After a 6 h incubation in the presence of Con A at 37°C in a humidified atmosphere with 5% CO<sub>2</sub> or 96 h with one of the antigens, the cell-free supernatant was removed and stored at -20°C until analysis for the IFN-gamma concentration by ELISA.

#### ***4.3.10 Cytokine ELISA***

To determine the IFN-gamma and interleukin 12 (IL-12) concentrations in the cell-free culture supernatants, 96-well Nunc maxisorp immunoplates (Life Technologies) were coated overnight at 4°C with a mouse anti-bovine IFN-gamma monoclonal antibody (mAb) (CC330; AbD Serotec) at 5 µg/ml or a mouse anti-bovine IL-12 mAb at 2.5 µg/ml (CC301; AbD Serotec), respectively. The plates were blocked for 2 h at room temperature (RT) with PBS containing 0.05% Tween<sup>®</sup> 20 and 3% non-fat milk powder. In subsequent steps we added, each for 1 h at RT, 100 µl of a cell culture supernatant 1:2 in PBS supplemented with 0.05% Tween<sup>®</sup> 20 and 1% non-fat milk powder, a biotin-labelled mouse anti-bovine IFN-gamma mAb (CC302b; AbD Serotec) or biotin-labelled mouse anti-bovine IL-12 mAb (CC326; AbD Serotec) and horseradish peroxidase (HRP)-conjugated streptavidin (Serotec, Belgium). In between each step, plates were washed 5 times with PBS 0.05% Tween<sup>®</sup> 20. Then, a HRP substrate solution (3,3',5,5'-tetramethylbenzidine (TMB, Sigma – Aldrich)) was added for 30 min at RT in the dark. The reaction was stopped by adding 50µl H<sub>2</sub>SO<sub>4</sub> (2N) and the optical density (OD) was measured at 450 nm.

The IFN-gamma and IL-12 concentrations in the samples were calculated from the regression line calculated using DeltaSoft JV 2.1.2 software. This regression line was obtained by including in the tests 10-fold dilutions of recombinant ovine IFN-gamma or recombinant IL-12 starting from 4000 pg/ml and 320 u/ml, respectively. The recombinant ovine IFN-gamma and ovine IL-12 were prepared as a serum-free conditioned medium from transfected CHO cells, according to a protocol described for the production of recombinant ovine IL-4 (Hope et al., 2005). The recombinant ovine IFN-gamma was quantified using the recombinant bovine IFN-gamma standard sold by Endogen, Thermo-scientific (Rockford, USA) and the anti-bovine mAbs (AbD Serotec) clones CC330 and CC302b in a species cross-reactive ELISA (personal communication with Sean Wattedgedera, Moredun Research Institute).

## 4.4 Results

### 4.4.1 *Neospora caninum* antibodies

None of the experimental infected or control animals showed an IgG response against *N. caninum* at euthanasia.

### 4.4.2 Presence of *T. gondii* in tissues

The presence of *T. gondii* in the various tissues collected after euthanasia was evaluated by qPCR (Table 4.1). Parasite DNA could be found sporadically in tissues of two out of three sheep euthanized at 4 days post infection (dpi). These sheep had *T. gondii* DNA in their epithelium (enterocytes, lamina propria) and in the organized lymphoid tissue of the small intestine (PP) or the mesenterial lymph nodes draining the small intestine (MLN), mostly in the cranial parts. In one sheep euthanized at 4 dpi parasite DNA could be detected in peripheral blood mononuclear cells (PBMC). No *T. gondii* DNA could be detected in the third sheep, suggesting that the parasite had not yet invaded the gut at this time point, had already left this site, or that the parasitic load was still below the detection limit. In the two sheep euthanized at 8 dpi, more tissues were positive for the presence of *T. gondii* DNA. The epithelium and draining lymph nodes of both sheep were positive throughout the small intestine, the lamina propria and Peyer's patches in the duodenum. Parasite DNA was demonstrated in other parts of the small intestine, popliteal lymph nodes and spleen in only one sheep. The appearance of DNA in the systemic tissues indicates that dissemination had taken place. At 10 dpi a similar pattern was seen for the intestinal tissues and MLN, but at this time point the two euthanized animals showed parasite DNA in their popliteal lymph nodes, confirming the dissemination observed at day 8 in one sheep. The animal euthanized at 14 dpi, had parasite DNA in all its intestinal tissues with the highest amounts in the Peyer's patches and MLN. Popliteal lymph nodes and spleen were negative. In the three sheep killed one week later (21 dpi), the parasite load had dropped below the detection limit in most tissues. Parasite DNA could still be detected in two animals. One was positive for the presence of *T. gondii* DNA in its MLN only, with the greatest amounts observed in the jejunal MLN. The other animal had low levels of parasite DNA in its jejunal epithelium and duodenal lamina propria. The third sheep tested completely negative. This suggests that either most of

the parasites had left the intestinal and lymphoid tissues or were cleared from these tissues at this time point.

When comparing the parasitic load in the tissues of the four animals euthanized at 8 and 10 dpi, a trend could be observed in cranial versus caudal intestinal tissues. For each of the four animals, one or more of the tested duodenal tissues or its associated draining lymph node, showed the highest or second highest amount of parasite DNA, while the tested ileal tissues often showed the lowest amount. In 5 of 12 ileal tissues samples no parasite DNA could be detected at all. Overall, the ileal Peyer's patches showed the lowest amount of parasite DNA. These data suggest that the parasite invades the gut primarily in the cranial small intestine shortly upon release from the cysts.

The two sham infected animals remained *T. gondii*-free in all tissues.

**Table 4.1:** Presence of *T. gondii* DNA in different small intestinal tissues, lymph nodes and blood of sheep orally infected with 3000 *T. gondii* PRU tachyzoites and euthanized at different days post infection (dpi).

Tissue		Parasite load as determined by RT qPCR and expressed as log 10 of parasite DNA/10 <sup>6</sup> cells.											
		DPI	4			8		10		14	21		
		Animals	Sh1	Sh2	Sh3	Sh4	Sh5	Sh6	Sh7	Sh8	Sh9	Sh10	Sh11
Duodenal E			0,36	nd	nd	3,06	-2,26	-3,06	-2,23	-2,90	nd	nd	nd
Duodenal LP			-0,43	nd	nd	2,88	-1,60	-1,46	-2,59	2,74	nd	nd	-1,58
Duodenal PP			nd	0,11	nd	2,26	-1,15	-2,68	-0,74	4,09	nd	nd	nd
Jejunal E			nd	nd	nd	2,23	-0,64	nd	-2,67	-1,54	nd	nd	-0,67
Jejunal LP			nd	nd	nd	nd	-0,02	-1,67	nd	-1,96	nd	nd	nd
Jejunal PP			nd	nd	nd	nd	-1,23	nd	-2,07	3,40	nd	nd	nd
Ileal E			nd	-0,16	nd	0,87	-1,44	nd	-3,29	-1,86	nd	nd	nd
Ileal LP			nd	nd	nd	nd	-0,44	-1,91	-4,00	-2,10	nd	nd	nd
Ileal PP			nd	nd	nd	nd	-2,00	nd	nd	2,95	nd	nd	nd
Duodenal MLN			0,34	nd	nd	1,95	-0,31	-1,20	-0,77	2,76	0,05	nd	nd
Jejunal MLN			2,63	nd	nd	1,45	-0,51	-0,99	-0,94	3,67	8,37	nd	nd
Ileal MLN			nd	nd	nd	2,44	-0,72	-2,87	-1,06	3,19	-1,29	nd	nd
Peripheral blood MC			0,45	nd	nd	nd	nd	nd	-1,68	-1,30	nd	nd	nd
Popliteal LN			nd	nd	nd	nd	-1,54	-1,66	-0,89	nd	nd	nd	nd
Splenocytes			nd	nd	nd	nd	-2,49	nd	-2,00	nd	nd	nd	nd

Sh = sheep. E = enterocytes. LP = lamina propria. PP = Peyer's patches. MLN = mesenteric lymph nodes. MC = mononuclear cells. LN = lymph node. nd = not detected

The grey scale reflects ranges in parasite load: white = nd to -3; light gray = -3 to -1; medium gray = -1 to 0; dark gray = 0 to 2; black = starting from 2

### 4.4.3 Humoral immune response

Serum samples were collected on a daily basis to determine the kinetics of the antibody response after the oral infection. *Toxoplasma*-specific IgM and IgG IFA's as well as TLA, rGRA7 and rROP2-specific IgG, IgG1, IgG2 and IgA ELISAs were performed. Data of the TLA and rROP2-specific IgG ELISAs are presented in Figure 4.1. The data of the rGRA7 ELISA did not give meaningful responses. Therefore, these data are not included in this chapter. The cut-off values for the other ELISAs were as follows: IgG: TLA: 0.117; rROP2: 0.167; IgG1: TLA: 0.321, rROP2: 0.306; IgG2: TLA: 0.348, rROP2: 0.159; IgA: TLA: 0.071, rROP2: 0.113

*Toxoplasma*-specific IgM was detected for the first in three out of the six not euthanized sheep at 9 dpi (3/6), two out of four at 12 dpi (2/4) and two out of three at 15 dpi (2/3). In one out of the three sheep, euthanized 3 weeks after infection, no *Toxoplasma*-specific IgM could be detected. IgG was detected by IFA in one sheep at 9 dpi (1/6) and in another at 12 dpi (1/4). These animals remained seropositive until euthanasia.

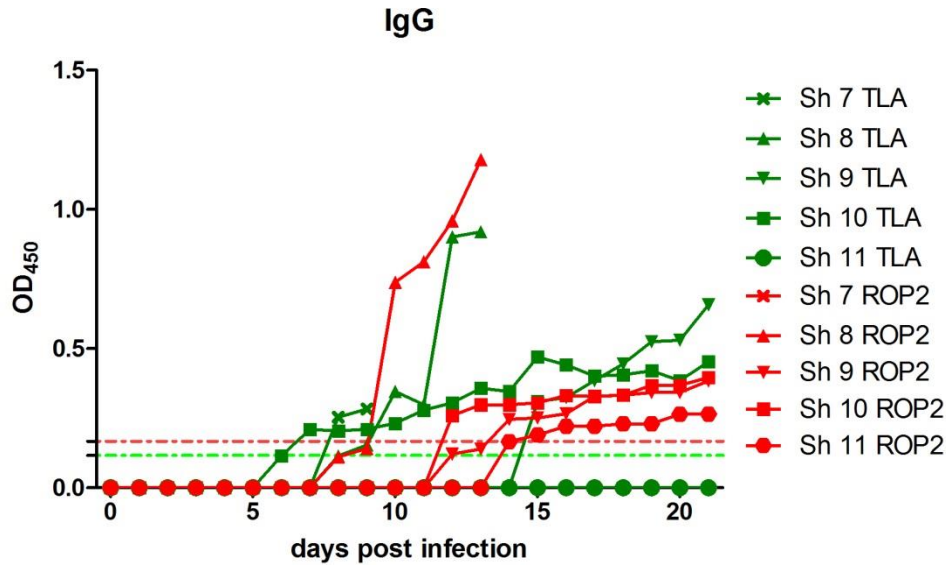
In the TLA ELISA, the *T. gondii*-specific IgG levels rose from 7, 8, 9 and 15 dpi onwards, each time in one animal and remained present in these animals until euthanasia (Fig. 4.1). Interestingly, in one animal no TLA-specific IgG response could be observed although its tissues were positive for the presence of parasite DNA at euthanasia, 21dpi. TLA-specific IgG1 appeared in one sheep 15 dpi and in another sheep 17 dpi. TLA-specific IgG2 appeared in the same two animals, but slightly earlier, namely on 12 and 15 dpi.

In the rROP2 ELISA, *T. gondii*-specific IgG appeared in four out of the five animals at 10, 12 and 14 dpi (Fig. 4.1). Interestingly, two sheep showed an IgG response against either TLA or rROP2, but not against both. Sh11 in which no TLA-specific serum IgG could be detected, showed a serum IgG response against rROP2 from 16 dpi on, while Sh7, with a response against TLA at 8 dpi, did not react against rROP2. Although the *T. gondii*-specific IgG1 and IgG2 responses against rROP2 were comparable with those against TLA, they showed a slightly delayed increase. Furthermore, one extra animal showed rROP2-specific antibodies.

*Toxoplasma*-specific serum IgA was not detected in any sheep.

The two sham-infected control sheep neither showed *T. gondii*-specific antibodies in IFA, nor in ELISA.





**Figure 4.1:** TLA and rROP2-specific IgG responses of sheep infected with *T. gondii* tissue cysts in function of time measured by ELISA. The dashed green and red lines indicate the cut-off values defined with pre-immune sera for TLA and rROP2, respectively.

#### 4.4.4 Interferon-gamma and interleukin-12 cytokine response

At euthanasia, MCs were isolated from blood, Peyer's patches, MLN, popliteal LN and spleen and restimulated with rGRA7, rEC2, rMIC3 and total lysate antigen (TLA) to determine the antigen-specific IFN-gamma and/or IL-12 responses.

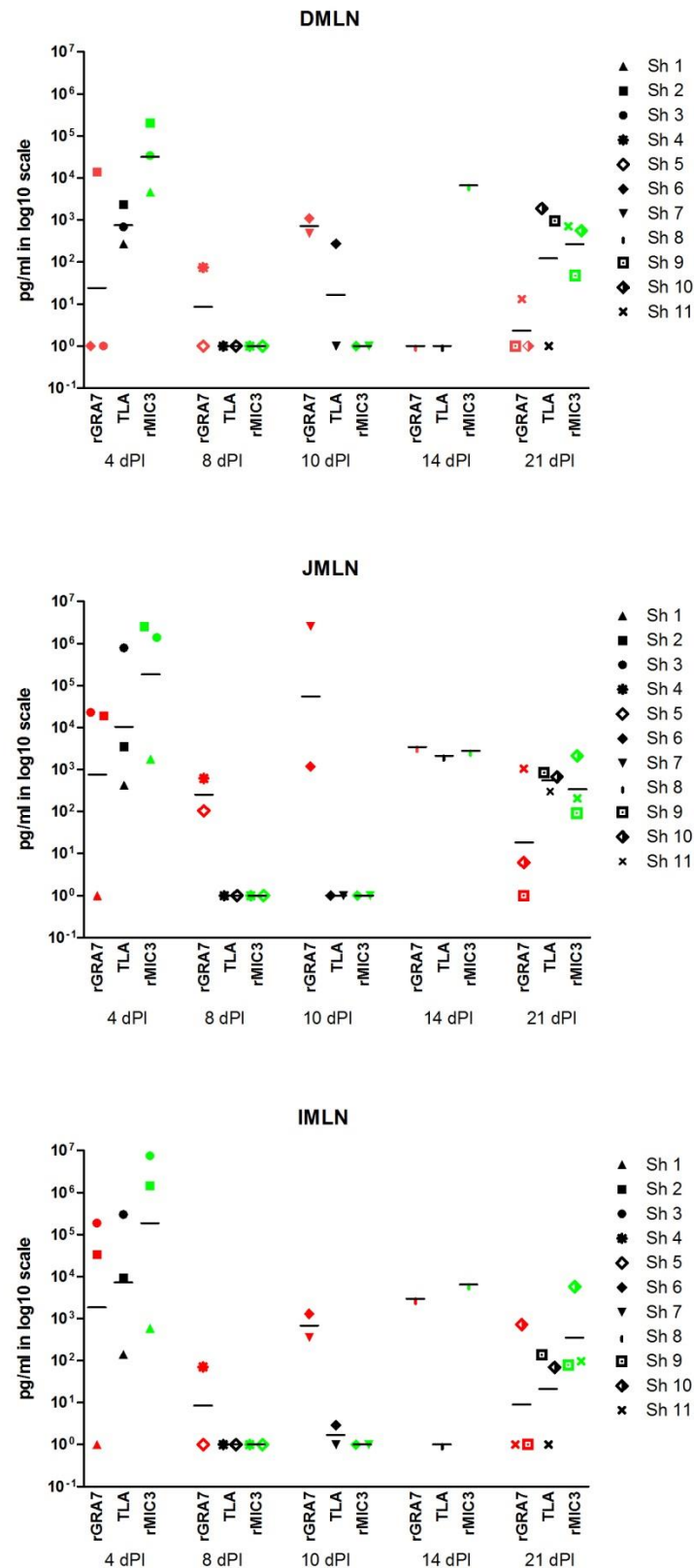
At 4 dpi, TLA, rEC2 and rMIC3 consistently induced high IFN-gamma concentrations in the MLN cultures of all 3 animals, with the highest responses against rMIC3 (DMLN: 80,637 ng/ml (mean)  $\pm$  61,951 (SEM); JMLN: 1,313,771 ng/ml  $\pm$  735,659; IMLN: 2,963,760 ng/ml  $\pm$  2,275,224); followed by rEC2 (DMLN: 5,400 ng/ml  $\pm$  2,204; JMLN: 129,964 ng/ml  $\pm$  119,802; IMLN: 298,194 ng/ml  $\pm$  155,314) and TLA (Fig. 4.2). In contrast, rGRA7 induced less consistently IFN-gamma (Fig. 4.2). Two out of three animals showed IFN-gamma production in jejunal and ileal MLN and one of them in duodenal MLN too (Fig. 4.2). IFN-gamma was also induced in popliteal LN and spleen, but mainly by rMIC3 and to a lesser extent by TLA (Fig 4.4). Responses in PP (Fig 4.3) and PBMC were absent.

At 8 and 10 dpi, the IFN-gamma pattern was different in that low or no responses were detectable in MLN (Fig. 4.2). Indeed, neither rEC2 nor rMIC3 induced IFN-gamma responses in the MLN cell cultures, whereas a low response was observed in one animal at 10 dpi. In contrast, rGRA7 stimulated IFN-gamma in MLN of all four animals with the lower concentrations at 8 dpi (Fig. 4.2). More IFN-gamma response occurred in PP (Fig. 4.3),

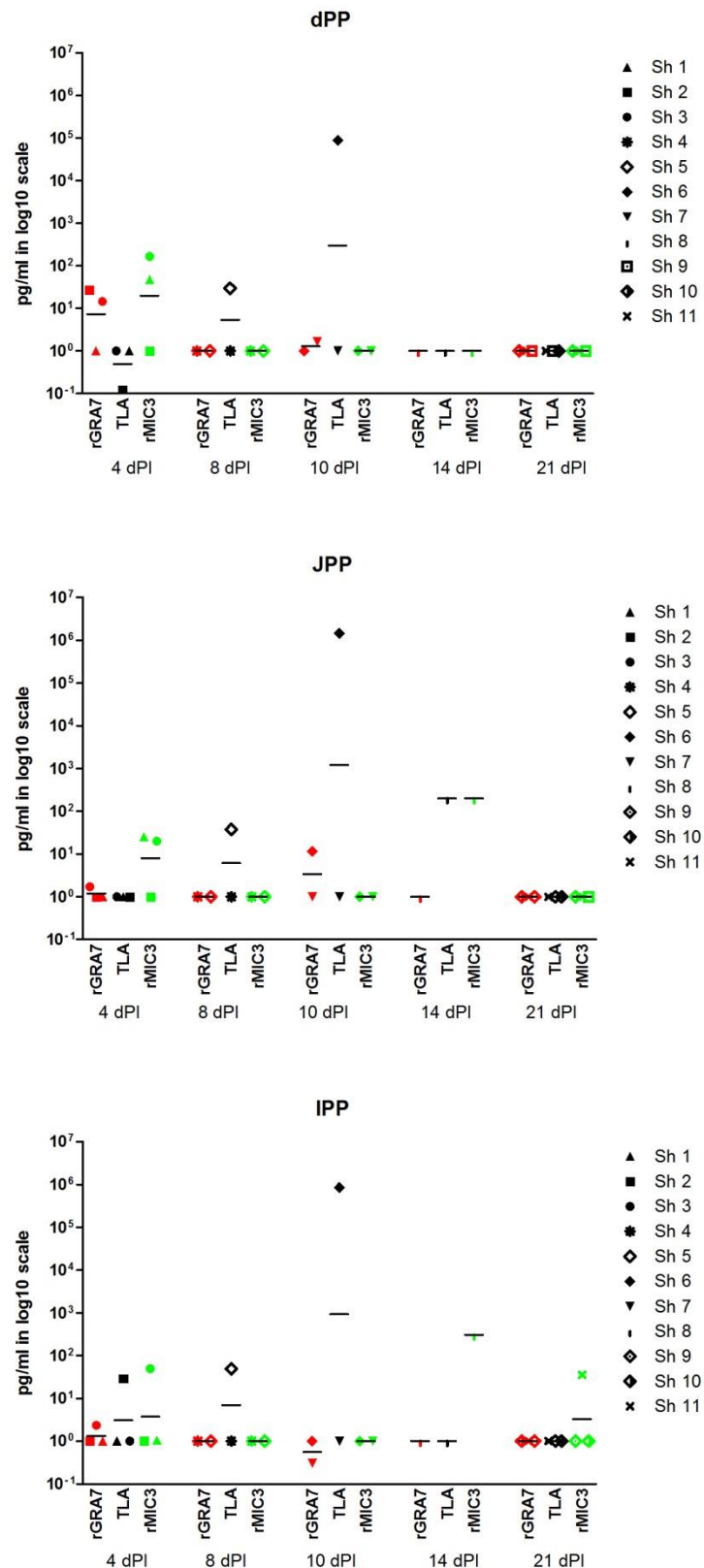
PBMC and popliteal LN (Fig. 4.4) cultures, with a higher response after TLA stimulation than after rGRA7 stimulation. Only TLA induced IFN-gamma in PP cell cultures of all three small intestinal sites in two out of four animals. The same two animals showed TLA-induced IFN-gamma in popliteal LN and PBMC cultures. rGRA7-induced IFN-gamma production was seen in popliteal LN cell cultures of three out of four animals, and in PBMC and spleen cell cultures in two out of four animals, but not or very weak in PP cell cultures.

At 14 and 21 dpi, only rMIC3 consistently induced high IFN-gamma in MLN cell cultures of all four animals, whereas the other antigens induced IFN-gamma in 50 to 67% of the cultures. These responses were less pronounced than at 4 dpi. In PP, popliteal LN and PBMC cultures rGRA7 and TLA induced responses were mostly absent, whereas responses could be seen in spleen cell cultures of three out of four animals for TLA (one sheep euthanatized at 14 dpi and two at 21 dpi) and of two out of four animals for rMIC3 and for rEC2 (at 21 dpi).

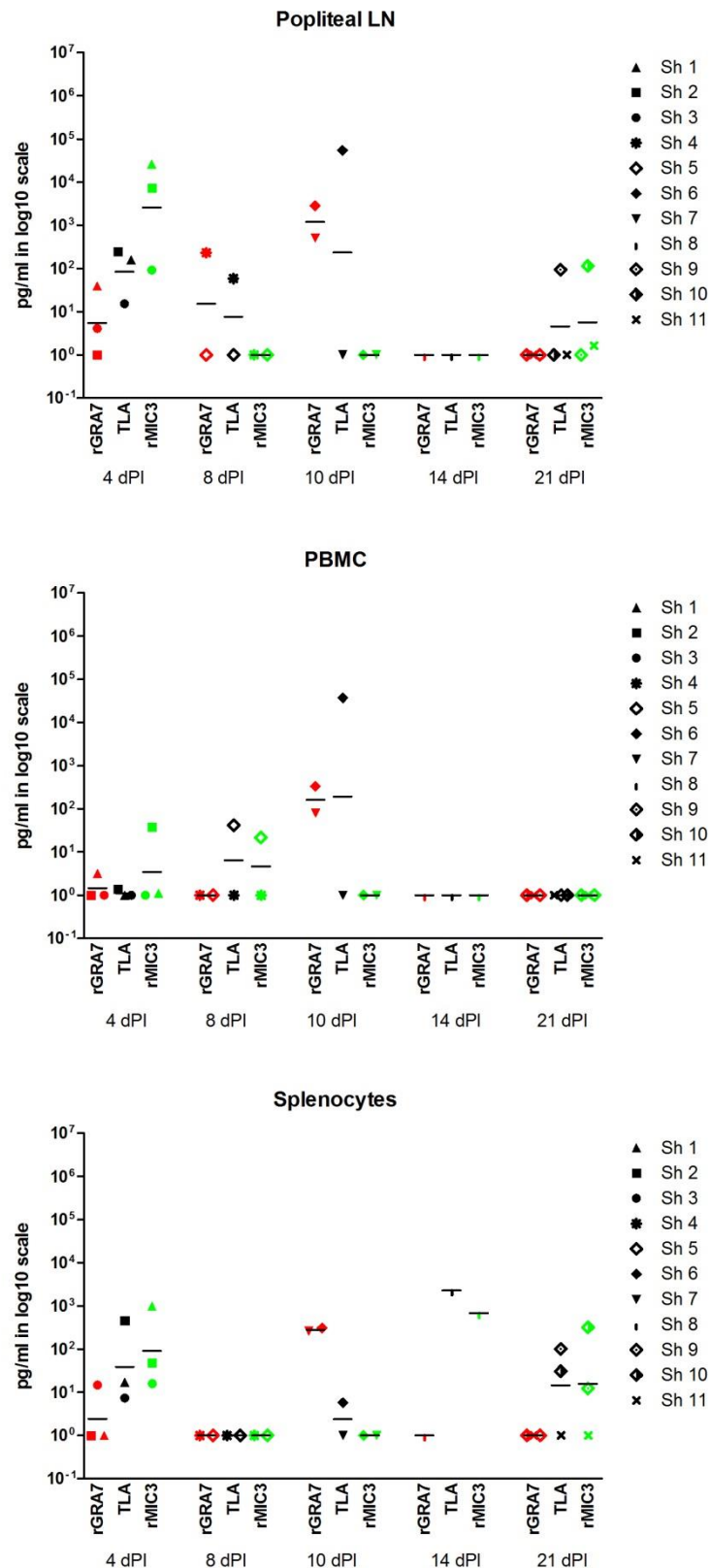
There was no induction of IFN-gamma in the MCs of the control animal.



**Figure 4.2:** IFN-gamma responses (log<sub>10</sub> scale) in duodenal (D), jejunal (J) and ileal (I) mesenteric lymph nodes (MLN), against rGRA7, TLA and rMIC3 in function of time following oral infection with *T. gondii* tissue cysts in sheep. The lines present the mean OD-value at each time point.



**Figure 4.3:** IFN-gamma responses (log<sub>10</sub> scale) in duodenal (D), jejunal (J) and ileal (I) Peyer's patches (PP), against rGRA7, TLA and rMIC3 in function of time following oral infection with *T. gondii* tissue cysts in sheep. The lines present the mean OD-value at each time point.

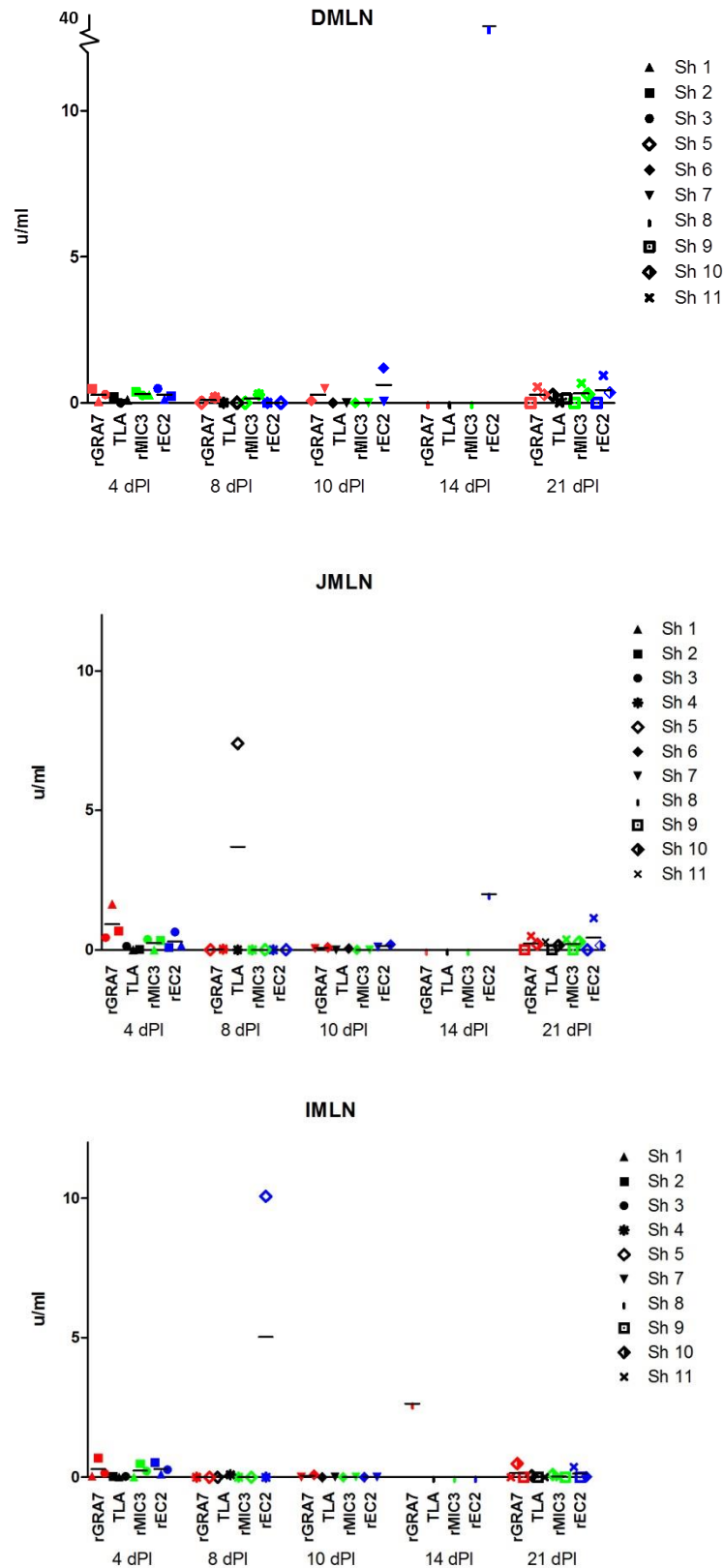


**Figure 4.4:** IFN-gamma responses (log<sub>10</sub> scale) in popliteal LN, PBMC and splenocytes against rGRA7, TLA and rMIC3 in function of time following oral infection with *T. gondii* tissue cysts in sheep. The lines present the mean OD-value at each time point.

Similar to IFN-gamma, the IL-12 production (Fig. 4.5) at day 4 pi was most consistently induced in the MLN cell cultures. However, whereas rGRA7 was the weakest inducer of IFN-gamma, it was the best inducer of IL-12 ( $0.50 \text{ u/ml} \pm 0.16 \text{ (SEM)}$ ), followed by rEC2 ( $0.29 \text{ u/ml} \pm 0.07 \text{ (SEM)}$ ), rMIC3 ( $0.26 \text{ u/ml} \pm 0.06 \text{ (SEM)}$ ) and TLA ( $0.06 \text{ u/ml} \pm 0.02 \text{ (SEM)}$ ). TLA induced low amounts of IL-12 in the DMLN of one animal and in the JMLN of another. Besides, low amounts of IL-12 were induced in the PP by rMIC3 in two animals and by TLA in the third animal and by rEC2 in the popliteal LN of one animal.

At 8 and 10 dpi, the responses of MLN were in general lower except for one animal in which TLA induced  $7.4 \text{ u/ml}$  in jejunal and rEC2  $10.1 \text{ u/ml}$  in ileal MLN. At least one antigen induced IL-12 in at least one of the MLN cell cultures of each animal, although none of the antigens could consistently induce IL-12 responses in the MLN cell cultures. The same was seen for the PP cell cultures. In popliteal LN cell cultures and spleen, IL-12 was produced following restimulation with different antigens in two of the 4 animals. PBMCs remained IL-12 negative. Accordingly, at these time points more variation was seen in the IL-12 response than in the IFN-gamma response. At 14 dpi, IL-12 was induced by rEC2 in cell cultures of all tissues except for the spleen. High amounts were observed in duodenal MLN ( $40.9 \text{ u/ml}$ ) and very high in popliteal LN cell cultures ( $635.6 \text{ u/ml}$ ). None of the other antigens induced IL-12 in MLN cell cultures. For the PP, rGRA7 and rMIC3 induced IL-12 in duodenal, rEC2 in jejunal and rMIC3 and TLA in ileal PP cell cultures. rEC2 as well as TLA induced also high IL-12 concentrations in the popliteal LN cell cultures ( $32.8 \text{ u/ml}$ ). Recombinant MIC3, rGRA7 and TLA induced IL-12 in PBMCs and splenocytes ( $1.7 - 6.1 \text{ u/ml}$ ). So, in particular the IL-12 response in popliteal LN and PBMCs gave a noteworthy difference with the IFN-gamma response.

At 21 dpi, neither in the popliteal LN nor in the PBMCs, IL-12 was detectable. On the other hand, IL-12 was induced by rEC2 and rMIC3 in the splenocyte culture of one animal, and was induced, in very low concentrations, by the recombinant antigens and TLA in most duodenal and jejunal MLN cultures and even less in the ileal MLN cultures.



**Figure 4.5:** IL-12 response (u/ml) in duodenal (D), jejunal (J) and ileal (I) mesenteric lymph nodes (MLN), against rGRA7, TLA and rMIC3 in function of time following oral infection with *T. gondii* tissue cysts in sheep. The lines present the mean OD-value at each time point.

## 4.5 Discussion

*T. gondii* seropositive sheep are probably latently infected for life and harbour tissue cysts in various tissues. Therefore, the consumption of raw or undercooked meat from seropositive animals is a high risk for humans (Buxton et al., 2007b). In the present study a type II strain was used for experimental infection of sheep since type II strains are most commonly found in animals in Europe and are most commonly identified as a cause of human toxoplasmosis world-wide (Sibley et al., 2009). A commercially available, live intramuscular vaccine (Toxovax®, MSD Animal Health) can prevent congenital toxoplasmosis in sheep. However, no current vaccine can prevent infection, suggesting that new vaccination strategies are needed. Induction of a mucosal immune response would be of interest since sheep become infected orally. Therefore, an important research goal is to identify the acute events in the small intestine following oral infection (Esteban-Redondo and Innes, 1997; Tenter et al., 2000). Whereas several natural and experimental *T. gondii* infections have been performed in sheep, none focused in the same detail on the initial phase of the infection at the intestinal mucosa and lymphoid tissues and its local cytokines response as we did in our study (Benavides et al., 2011; Buxton et al., 2007a, b; Dubey, 2009a; Falcon and Freyre, 2009; Gutierrez et al., 2010; Innes et al., 2009b). In our study, sheep were orally infected with 3000 tissue cysts. Because infectious oocysts are more difficult to obtain in sufficient quantities, lead to environmental contamination and are a serious risk for laboratory personnel, tissue cysts were used even though oral infection in sheep mostly occurs via ingestion of oocysts during grazing (Race et al., 1998). Nevertheless infection with tissue cysts can occur, although rare, when sheep eat the placenta or aborted tissues from ewes that aborted as a result of toxoplasmosis (Dubey, 1987). As mentioned above, only few studies analyzed the immune response in sheep, three of them after oral inoculation with oocysts (Benavides et al., 2011; Coughlan et al., 1995; Gutierrez et al., 2010) and four following intraperitoneal injection with tachyzoites of the RH strain or the S48 strain (Buxton et al., 1994; Innes et al., 1995a; Innes et al., 1995b; Wastling et al., 1993). One other study examined the immune response following a subcutaneous inoculation with tissue cysts (McColgan et al., 1987). *T. gondii* bradyzoites entered preferentially the cranial small intestine around 4 dpi. Most likely, infection occurs at the same site when oocysts are given orally. Indeed, Dubey and Sharma (1980) found that excystation of oocysts and the release of sporozoites takes place in the small intestine.



Since parasites were detected simultaneously in epithelial cells, lamina propria, MLN and blood in one sheep at 4 dpi, our findings suggest that some parasites most likely directly passed the epithelial barrier to reach the MLN and the blood. The early detection of parasite DNA in blood in our study could be a consequence of the use of tissue cysts for oral infection; bradyzoites released from tissue cysts might pass the epithelial layer earlier than sporozoites released from oocysts. Indeed, Dubey and Sharma (1980) found that parasitemia occurs between 6 and 11 days after oral inoculation of sheep with oocysts.

Four days after infection sheep were still seronegative in IFA as well as in TLA and rROP2 ELISA. Although the mesenteric lymphocytes and splenocytes produce more IFN-gamma in response to antigen stimulation 4 dpi than later during infection, this response is too early to be derived from antigen-specific CD4<sup>+</sup> and/or CD8<sup>+</sup> T lymphocytes. A T- cell independent IFN-gamma production by NK-cells (Sher et al., 1993) has been described in mice and is dependent on the production of IL-12 and tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) by macrophages (Gazzinelli et al., 1993). However, in the present study, a generalized but low IL-12 production was measured in the MLN cultures at 4 dpi. During this early infection stage, the parasite triggers several components of the innate immune system resulting in the release of cytokines such as IL-12 and IFN-gamma by macrophages, NK cells, dendritic cells as well as neutrophils. Previous studies in mice have demonstrated that IL-12 production is critical in inducing an early IFN-gamma production, which most likely plays an important role in the host-resistance (Bhopale, 2003; Gazzinelli et al., 1994). Indeed, in IL-12 knockout mice, severely decreased IFN-gamma levels led to an uncontrolled replication of *T. gondii* tachyzoites, which caused the early death of the IL-12 deficient mice (Vossenkamper et al., 2004). While triggering the innate immune system, parasite antigens are also taken up and presented by antigen-presenting cells, inducing a parasite-specific T-cell immunity (Denkers and Gazzinelli, 1998). At 8 dpi, parasite DNA was highly present in the tissues of the cranial small intestine and MLN and this remained so until 14 dpi, suggesting that replication of the parasite occurs in the small intestinal mucosa, mucosa-associated lymphoid tissues and associated draining lymph nodes. This finding is in accordance with the observations of Dubey and Sharma (1980) who found tachyzoites in the mesenteric lymph nodes of sheep from 7 dpi onwards. Similarly in mice, an increase in parasite burden occurs in PP, MLN, IE and LP between 4 and 9 dpi (Morampudi et al., 2011). Specific IgM and IgG antibodies appeared almost simultaneously at 9 dpi. Specific antibodies in the presence of complement can lyse extracellular tachyzoites (Bhopale, 2003). Parallel with the appearance of antibodies, a significant decrease in the IFN-gamma response and a decrease in the IL-12 response were

observed. Different antigens induced IL-12 responses in various gut tissues of different sheep, which was not the case for IFN-gamma. Recombinant GRA7 induced quite consistent IFN-gamma responses in MLN lymphocyte cultures of the four animals tested at 8 and 10 dpi, whereas TLA clearly induced IFN-gamma in the PP lymphocyte culture of two of the four euthanized animals. This suggests that various antigens are involved in the induction of the IFN-gamma responses in PP versus MLN. Homing of the activated T cells from both lymphoid tissues to other sites could explain why both antigen preparations induced responses in PBMC as well as in the popliteal LN cell cultures. Such homing at 8 dpi is consistent with the increase of IFN-gamma producing CD4<sup>+</sup> lymphocytes in efferent lymph of surgically cannulated experimentally infected lymph nodes at 11 dpi (Innes et al., 1995a). Our results suggest that a T cell independent response becomes replaced by an antigen-specific T lymphocyte response around 8 dpi. The finding that, in popliteal lymph nodes, spleen cell cultures and distant systemic lymphoid tissues, IFN-gamma and IL-12 responses can be induced by several antigens at 8 dpi supports the hypothesis that a systemic migration of the parasites occurs at that time.

Our observation that the parasite DNA disappears from the small intestine, popliteal LN, PBMC and spleen, 3 weeks after infection, indicates that the parasite load drops below the detection limit of the PCR. Indeed, a negative result in PCR does not imply that these tissues are completely free of parasite DNA. However, the finding that most of the tested tissues became PCR negative could be an indication that the parasite is either suppressed in these tissues, or is cleared from these tissues or otherwise is leaving these tissues between 2 and 3 weeks post infection. This coincided with an increase of IgG antibodies between 9 and 21 dpi. In previous studies, the immune response was studied following oral inoculation with oocysts or following intraperitoneal injection of tachyzoites of the RH strain (Tenter et al., 1992; Verma et al., 1989). Whereas antibodies were detectable 14 days after oral inoculation with 5.000 or 50.000 oocysts (Tenter et al., 1992), they only appeared 1 month after intraperitoneal injection of tachyzoites (Verma et al., 1989). In our study the oral inoculation with tissue cysts resulted in a more rapid antibody response. However, this response was similar to the one observed by Mc Colghan et al (1987), who observed appearance of IgG 9 days after experimental infection with tissue cysts. This again supports the hypothesis that the parasite passes the intestinal epithelial barrier more quickly after an oral infection with tissue cysts although it cannot be excluded that the earlier response was a result of strain differences (Dubey, 2009b; Dubey and Sharma, 1980; Esteban-Redondo and Innes, 1997). While the antibody response increased and the parasite seems to disappear from systemic lymphoid

tissues, the IL-12 response in these tissues decreased or became absent. This coincided with a decreased or negative antigen-specific IFN-gamma response in PP, PBMC and popliteal LN cell cultures. Interestingly, these observations are in accordance with the findings of Innes et al. (1995) who infected sheep subcutaneously with tachyzoites. IFN-gamma could not be detected anymore in the lymph from lymph nodes draining the subcutaneous region from 11 dpi onwards. At that time they noticed a switch from predominantly CD4<sup>+</sup> proliferating lymphocytes to CD8<sup>+</sup> proliferating lymphocytes. In our study, antigen-specific IFN-gamma responses were still present in MLN and spleen cell cultures 21 dpi. Since MLN not only drain the gut, but also the systemic sites such as the peritoneal cavity, and since the spleen is the secondary lymphoid organ draining the blood, both responses could play a role in controlling a systemic infection occurring at this stage (Innes et al., 1995a).

Although our experimental animal groups were too small to warrant firm conclusions, our data show a strong agreement with previous results. Further studies with larger number of animals are needed to confirm the obtained results and conclusions.

## 4.6 Conclusions

Our results indicate that parasites enter the cranial small intestine the first days after infection. Three weeks later *T. gondii* DNA could not be recovered anymore from the intestine. This coincided with the increase of IgG1 and IgG2 antibodies and a decrease of the antigen-specific IFN-gamma response in PP, PBMC and popliteal LN. The experiments support a collaborate role of humoral and cellular immunity in acute *T. gondii* infections in sheep.

## **Acknowledgements**

The work described herein was funded by the “Institute for the Promotion of Innovation through Science and Technology in Flanders (IWT-Vlaanderen) and the Federal Public Service, Health, Food Chain Safety and Environment.

GE is funded by the Scottish Government Rural and Environmental Science and Analytical Services Division (RESAS) and supported by the European Community’s Seventh Framework Programme (FP7, 2007-2013), Research Infrastructures action, under the grant agreement No. FP7-228394 (NADIR).

We are indebted to Dr. N. Gargano (Kenton Laboratories, Rome, Italy) for allowing us to work with the EC2 chimeric antigen.

## 4.7 References

- Aubert, D., Maine, G.T., Villena, I., Hunt, J.C., Howard, L., Sheu, M., Brojanac, S., Chovan, L.E., Nowlan, S.F., Pinon, J.M., 2000. Recombinant antigens to detect *Toxoplasma gondii*-specific immunoglobulin G and immunoglobulin M in human sera by enzyme immunoassay. *J Clin Microbiol* 38, 1144-1150.
- Benavides, J., Maley, S., Pang, Y., Palarea, J., Eaton, S., Katzer, F., Innes, E.A., Buxton, D., Chianini, F., 2011. Development of lesions and tissue distribution of parasite in lambs orally infected with sporulated oocysts of *Toxoplasma gondii*. *Vet Parasitol* 179, 209-215.
- Bhopale, G.M., 2003. Development of a vaccine for toxoplasmosis: current status. *Microbes Infect* 5, 457-462.
- Bivas-Benita, M., Laloup, M., Versteheyhe, S., Dewit, J., De Braekeleer, J., Jongert, E., Borchard, G., 2003. Generation of *Toxoplasma gondii* GRA1 protein and DNA vaccine loaded chitosan particles: preparation, characterization, and preliminary in vivo studies. *Int J Pharm* 266, 17-27.
- Blewett, D.A., Miller, J.K., Buxton, D., 1982. Response of immune and susceptible ewes to infection with *Toxoplasma gondii*. *Vet Rec* 111, 175-178.
- Blewett, D.A., Watson, W.A., 1983. The epidemiology of ovine toxoplasmosis. II. Possible sources of infection in outbreaks of clinical disease. *Br Vet J* 139, 546-555.
- Bonhomme, A., Maine, G.T., Beorchia, A., Burlet, H., Aubert, D., Villena, I., Hunt, J., Chovan, L., Howard, L., Brojanac, S., Sheu, M., Tyner, J., Pluot, M., Pinon, J.M., 1998. Quantitative immunolocalization of a P29 protein (GRA7), a new antigen of *toxoplasma gondii*. *J Histochem Cytochem* 46, 1411-1422.
- Buxton, D., 1998. Protozoan infections (*Toxoplasma gondii*, *Neospora caninum* and *Sarcocystis spp.*) in sheep and goats: recent advances. *Vet Res* 29, 289-310.
- Buxton, D., Maley, S.W., Wright, S.E., Rodger, S., Bartley, P., Innes, E.A., 2007a. Ovine toxoplasmosis: transmission, clinical outcome and control. *Parassitologia* 49, 219-221.
- Buxton, D., Maley, S.W., Wright, S.E., Rodger, S., Bartley, P., Innes, E.A., 2007b. *Toxoplasma gondii* and ovine toxoplasmosis: new aspects of an old story. *Vet Parasitol* 149, 25-28.
- Buxton, D., Thomson, K.M., Maley, S., Wastling, J.M., Innes, E.A., Panton, W.R., Nicoll, S., 1994. Primary and secondary responses of the ovine lymph node to *Toxoplasma gondii*: cell output in efferent lymph and parasite detection. *J Comp Pathol* 111, 231-241.
- Cardoso, L.S., Araujo, M.I., Goes, A.M., Pacifico, L.G., Oliveira, R.R., Oliveira, S.C., 2007. Polymyxin B as inhibitor of LPS contamination of *Schistosoma mansoni* recombinant proteins in human cytokine analysis. *Microb Cell Fact* 6, 1.

- Cenci-Goga, B.T., Rossitto, P.V., Sechi, P., McCrindle, C.M., Cullor, J.S., 2011. *Toxoplasma* in animals, food, and humans: an old parasite of new concern. *Foodborne pathogens and disease* 8, 751-762.
- Cook, A.J., Gilbert, R.E., Buffolano, W., Zufferey, J., Petersen, E., Jenum, P.A., Foulon, W., Semprini, A.E., Dunn, D.T., 2000. Sources of *toxoplasma* infection in pregnant women: European multicentre case-control study. European Research Network on Congenital Toxoplasmosis. *BMJ* 321, 142-147.
- Coughlan, S.N., Saman, E., Jacobs, D., Mercier, C., Cesbron-Delauw, M.F., Trees, A.J., 1995. Cellular and humoral immune responses to recombinant antigens in sheep infected with *Toxoplasma gondii*. *Parasite Immunol* 17, 465-468.
- Denkers, E.Y., Gazzinelli, R.T., 1998. Regulation and function of T-cell-mediated immunity during *Toxoplasma gondii* infection. *Clin Microbiol Rev* 11, 569-588.
- Dubey, J.P., 1987. *Toxoplasma gondii* cysts in placentas of experimentally infected sheep. *Am J Vet Res* 48, 352-353.
- Dubey, J.P., 2009a. History of the discovery of the life cycle of *Toxoplasma gondii*. *Int J Parasitol* 39, 877-882.
- Dubey, J.P., 2009b. Toxoplasmosis in sheep--the last 20 years. *Vet Parasitol* 163, 1-14.
- Dubey, J.P., Sharma, S.P., 1980. Parasitemia and tissue infection in sheep fed *Toxoplasma gondii* oocysts. *J Parasitol* 66, 111-114.
- Dubey, J.P.a.B., C. P., 1988. Toxoplasmosis of animals and man. CRC Press, Inc., Boca Raton, FL 33431, USA, 220 p.
- EFSA, 2007. Scientific opinion of the panel on Biological Hazards and Animal Health Animal Welfare on a request from the European Food Safety Authority (self mandate) to issue a scientific opinion on the review of the Community Summary Report on Trends and Sources of Zoonoses, Zoonotic Agents and Antimicrobial Resistance in the European Union in 2005. *EFSA Journal* 1-32 (<http://www.efsa.europa.eu/en/scdocs/doc/600.pdf>).
- Esteban-Redondo, I., Innes, E.A., 1997. *Toxoplasma gondii* infection in sheep and cattle. *Comp Immunol Microbiol Infect Dis* 20, 191-196.
- Falcon, J., Freyre, A., 2009. *Toxoplasma gondii*: prototype immunization of lambs against formation of muscle and brain cysts. *Vet Parasitol* 166, 15-20.
- Fischer, H.G., Stachelhaus, S., Sahm, M., Meyer, H.E., Reichmann, G., 1998. GRA7, an excretory 29 kDa *Toxoplasma gondii* dense granule antigen released by infected host cells. *Mol Biochem Parasitol* 91, 251-262.
- Gazzinelli, R.T., Denkers, E.Y., Sher, A., 1993. Host resistance to *Toxoplasma gondii*: model for studying the selective induction of cell-mediated immunity by intracellular parasites. *Infectious agents and disease* 2, 139-149.

- Gazzinelli, R.T., Wysocka, M., Hayashi, S., Denkers, E.Y., Hieny, S., Caspar, P., Trinchieri, G., Sher, A., 1994. Parasite-induced IL-12 stimulates early IFN-gamma synthesis and resistance during acute infection with *Toxoplasma gondii*. *J Immunol* 153, 2533-2543.
- Gutierrez, J., O'Donovan, J., Williams, E., Proctor, A., Brady, C., Marques, P.X., Worrall, S., Nally, J.E., McElroy, M., Bassett, H., Sammin, D., Buxton, D., Maley, S., Markey, B.K., 2010. Detection and quantification of *Toxoplasma gondii* in ovine maternal and foetal tissues from experimentally infected pregnant ewes using real-time PCR. *Vet Parasitol* 172, 8-15.
- Holec-Gasior, L., 2013. *Toxoplasma gondii* recombinant antigens as tools for serodiagnosis of human toxoplasmosis: current status of studies. *Clin Vaccine Immunol* 20, 1343-1351.
- Hope, J.C., Kwong, L.S., Thom, M., Sopp, P., Mwangi, W., Brown, W.C., Palmer, G.H., Wattegedera, S., Entrican, G., Howard, C.J., 2005. Development of detection methods for ruminant interleukin (IL)-4. *J Immunol Methods* 301, 114-123.
- Hunter, C.A., Subauste, C.S., Remington, J.S., 1994. The role of cytokines in toxoplasmosis. *Biotherapy* 7, 237-247.
- Innes, E.A., Bartley, P.M., Buxton, D., Katzer, F., 2009b. Ovine toxoplasmosis. *Parasitology* 136, 1887-1894.
- Innes, E.A., Panton, W.R., Sanderson, A., Thomson, K.M., Wastling, J.M., Maley, S., Buxton, D., 1995a. Induction of CD4+ and CD8+ T cell responses in efferent lymph responding to *Toxoplasma gondii* infection: analysis of phenotype and function. *Parasite Immunol* 17, 151-160.
- Innes, E.A., Panton, W.R., Thomson, K.M., Maley, S., Buxton, D., 1995b. Kinetics of interferon gamma production in vivo during infection with the S48 vaccine strain of *Toxoplasma gondii*. *J Comp Pathol* 113, 89-94.
- Jacobs, D., Vercammen, M., Saman, E., 1999. Evaluation of recombinant dense granule antigen 7 (GRA7) of *Toxoplasma gondii* for detection of immunoglobulin G antibodies and analysis of a major antigenic domain. *Clin Diagn Lab Immunol* 6, 24-29.
- James, G.T., 1978. Inactivation of the protease inhibitor phenylmethylsulfonyl fluoride in buffers. *Anal Biochem* 86, 574-579.
- Kijlstra, A., Meerburg, B., Cornelissen, J., De Craeye, S., Vereijken, P., Jongert, E., 2008. The role of rodents and shrews in the transmission of *Toxoplasma gondii* to pigs. *Vet Parasitol* 156, 183-190.
- Lind, P., Haugegaard, J., Wingstrand, A., Henriksen, S.A., 1997. The time course of the specific antibody response by various ELISAs in pigs experimentally infected with *Toxoplasma gondii*. *Vet Parasitol* 71, 1-15.
- Martrou, P., Pestre, M., Loubet, R., Nicolas, A., Malinvaud, G., 1965. La toxoplasmose congénitale (note concernant un cas mortel). *Limousin Med* 53, 3-7.

- McColgan, C., Buxton, D., Miller, H.R., 1987. Studies on ovine efferent lymph following infection with *Toxoplasma gondii*. J Comp Pathol 97, 695-703.
- Morampudi, V., De Craeye, S., Le Moine, A., Detienne, S., Braun, M.Y., D'Souza, S., 2011. Partial depletion of CD4(+)CD25(+)Foxp3(+) T regulatory cells significantly increases morbidity during acute phase *Toxoplasma gondii* infection in resistant BALB/c mice. Microbes Infect 13, 394-404.
- Morley, E.K., Williams, R.H., Hughes, J.M., Thomasson, D., Terry, R.S., Duncanson, P., Smith, J.E., Hide, G., 2008. Evidence that primary infection of Charollais sheep with *Toxoplasma gondii* may not prevent foetal infection and abortion in subsequent lambings. Parasitology 135, 169-173.
- Nigro, M., Martin, V., Kaufer, F., Carral, L., Angel, S.O., Pszeny, V., 2001. High level of expression of the *Toxoplasma gondii*-recombinant Rop2 protein in Escherichia coli as a soluble form for optimal use in diagnosis. Molecular biotechnology 18, 269-273.
- Pappas, G., Roussos, N., Falagas, M.E., 2009. Toxoplasmosis snapshots: global status of *Toxoplasma gondii* seroprevalence and implications for pregnancy and congenital toxoplasmosis. Int J Parasitol 39, 1385-1394.
- Rodger, S.M., Maley, S.W., Wright, S.E., Mackellar, A., Wesley, F., Sales, J., Buxton, D., 2006. Role of endogenous transplacental transmission in toxoplasmosis in sheep. Vet Rec 159, 768-772.
- Selseleh, M., Modarressi, M., Shojaei, S., Mohebbi, M., Eshraghian, M., Selseleh, M., Keshavarz, H., 2013. Brain Tissue Cysts in Infected Mice with RH-Strain of *Toxoplasma gondii* and Evaluation of BAG1 and SAG1 Genes Expression. Iranian journal of parasitology 8, 40-46.
- Sher, A., Oswald, I.P., Hieny, S., Gazzinelli, R.T., 1993. *Toxoplasma gondii* induces a T-independent IFN-gamma response in natural killer cells that requires both adherent accessory cells and tumor necrosis factor-alpha. J Immunol 150, 3982-3989.
- Sibley, L.D., Khan, A., Ajioka, J.W., Rosenthal, B.M., 2009. Genetic diversity of *Toxoplasma gondii* in animals and humans. Philos Trans R Soc Lond B Biol Sci 364, 2749-2761.
- Tenter, A.M., Heckeroth, A.R., Weiss, L.M., 2000. *Toxoplasma gondii*: from animals to humans. Int J Parasitol 30, 1217-1258.
- Tenter, A.M., Vietmeyer, C., Johnson, A.M., 1992. Development of ELISAs based on recombinant antigens for the detection of *Toxoplasma gondii*-specific antibodies in sheep and cats. Vet Parasitol 43, 189-201.
- Vande Walle, K., De Zutter, L., Cox, E., 2011. Oral infection with a Shiga toxin-negative *Escherichia coli* O157:H7 strain elicits humoral and cellular responses but does not protect sheep from colonisation with the homologous strain. Vet Microbiol 148, 317-322.
- Vandenbroeck, K., Nauwynck, H., Vanderpooten, A., Van Reeth, K., Goddeeris, B., Billiau, A., 1998. Recombinant porcine IFN-gamma potentiates the secondary IgG and IgA responses to an inactivated suid herpesvirus-1 vaccine and reduces postchallenge weight loss and fever in pigs. J Interferon Cytokine Res 18, 739-744.



- Vercammen, M., Scorza, T., Huygen, K., De Braekeleer, J., Diet, R., Jacobs, D., Saman, E., Verschueren, H., 2000. DNA vaccination with genes encoding *Toxoplasma gondii* antigens GRA1, GRA7, and ROP2 induces partially protective immunity against lethal challenge in mice. *Infect Immun* 68, 38-45.
- Verhelst, D., De Craeye, S., Dorny, P., Melkebeek, V., Goddeeris, B., Cox, E., Jongert, E., 2011. IFN-gamma expression and infectivity of *Toxoplasma* infected tissues are associated with an antibody response against GRA7 in experimentally infected pigs. *Vet Parasitol* 179, 14-21.
- Verma, S.P., Bhardwaj, R.M., Gautam, O.P., 1989. Application of countercurrent immunoelectrophoresis (CIEP) in the sero-diagnosis of *Toxoplasma* antibodies in sheep. *J. Vet. Parasitol.*, 61-62.
- VIDA, 2009. Veterinary Investigation Surveillance Report
- Vossenkamper, A., Struck, D., Alvarado-Esquivel, C., Went, T., Takeda, K., Akira, S., Pfeffer, K., Alber, G., Lochner, M., Forster, I., Liesenfeld, O., 2004. Both IL-12 and IL-18 contribute to small intestinal Th1-type immunopathology following oral infection with *Toxoplasma gondii*, but IL-12 is dominant over IL-18 in parasite control. *Eur J Immunol* 34, 3197-3207.
- Wastling, J.M., Nicoll, S., Buxton, D., 1993. Comparison of two gene amplification methods for the detection of *Toxoplasma gondii* in experimentally infected sheep. *J Med Microbiol* 38, 360-365.
- Wingstrand, A., Lind, P., Haugegaard, J., Henriksen, S.A., Bille-Hansen, V., Sorensen, V., 1997. Clinical observations, pathology, bioassay in mice and serological response at slaughter in pigs experimentally infected with *Toxoplasma gondii*. *Vet Parasitol* 72, 129-140.



## **CHAPTER 5**

### ***INTERFERON GAMMA EXPRESSION AND INFECTIVITY OF *TOXOPLASMA* INFECTED TISSUES FROM EXPERIMENTALLY INFECTED SHEEP IN COMPARISON WITH PIGS***

*Based on:* Verhelst, D., De Craeye, S., Dorny, P., Melkebeek, V., Goddeeris, B., Cox, E., Jongert, E., 2011. IFN-gamma expression and infectivity of *Toxoplasma* infected tissues are associated with an antibody response against GRA7 in experimentally infected pigs. *Vet Parasitol* 179, 14-21.

Verhelst, D., De Craeye, S., Jennes, M., Dorny, P., Goddeeris, B., Cox, E., 2014. Interferon gamma expression and infectivity of *Toxoplasma* infected tissues from experimentally infected sheep in comparison with pigs. Submitted



## 5.1 Abstract

Livestock animals are a potential risk for transmission of toxoplasmosis to humans. Sheep and pigs still remain an important source because their meat is often consumed undercooked, which is increasingly being regarded as a major route of infection in many countries. Moreover, porcine tissues are processed in many food products.

In the current study, the IFN-gamma (T-helper 1 cells), IL-4 (Th2 cells) and IL-10 mRNA (Treg cells) expression by blood mononuclear cells, and the serum antibody response against *Toxoplasma gondii* total lysate antigen, recombinant *T. gondii* GRA1, rGRA7, rMIC3 and rEC2, a chimeric antigen composed of MIC2, MIC3 and SAG1, was studied in sheep the first two months after a *T. gondii* infection and compared with these responses in pigs. At the end of this period, the parasite distribution in heart, brain and two skeletal muscles was studied in sheep and pigs.

Whereas the parasite distribution was similar in sheep and pigs, the antibody response differed considerably. In sheep, antibodies appeared against all tested *T. gondii* antigens, but mainly against rGRA7, rMIC3<sub>234-307</sub> and TLA, whereas in pigs only rGRA7-specific antibodies could be demonstrated. Also, the cytokine response differed. Both in sheep and pigs an IFN-gamma response occurred, which seemed to be a slightly more pronounced in sheep. In sheep, also IL-10 and IL-4 mRNA expression increased, but later than IFN-gamma and with more variation. However, in pigs no such increase was seen.

Concerning diagnosis of a *T. gondii* infection, results indicate that, in live sheep and pigs, serum antibodies for a rGRA7 ELISA, and in euthanized animals, heart tissue for bioassay and qPCR, are the best biological samples to demonstrate presence of *T. gondii* infection.

## 5.2 Introduction

*Toxoplasma gondii*, an obligate intracellular protozoan parasite, is one of the most common parasitic zoonoses worldwide (Tenter et al., 2000). The parasite can establish a chronic infection in animals and humans. By estimation,  $5 \times 10^8$  people worldwide or one third of the world population has been infected (Denkers and Gazzinelli, 1998; Montoya and Liesenfeld, 2004). Livestock animals are a potential risk for transmission of toxoplasmosis to humans. Consumption of raw or undercooked meat is regarded as a major route of infection in many countries (Cook et al., 2000; Kijlstra and Jongert, 2008). Seroprevalence studies showed that in certain countries 90% of the sheep are infected (Dubey, 2009). In contrast the

seroprevalence in pigs is very low to almost zero (Kijlstra et al., 2004). Nevertheless, sheep and pigs still remain an important source of human infection because their meat is often consumed undercooked and porcine tissues are processed in many food products.

Following oral ingestion of oocysts/tissue cysts by sheep, the parasites transform into tachyzoites, which invade the small intestine, where they rapidly replicate in the epithelial cells (Chapter 4). Simultaneously, they spread to other gut-associated tissues. This is accompanied with the induction of cellular and humoral immune responses. Cellular responses occur first with the interferon gamma (IFN-gamma) responses peaking in mesenteric lymph nodes (MLN) and splenocytes 4 days post infection (dpi) and the IL-12 responses in MLN most-likely as a result of innate immune responses (Chapter 4). A second peak occurs 1 to 3 weeks later and is most-likely due to T-cell responses (Chapter 4). These T-cell responses are necessary for the survival of the host and as a result the tachyzoite stage of the parasite transforms into the bradyzoite stage, which escapes the immune system and persists in cysts in the host tissues (Denkers and Gazzinelli, 1998). Since the parasite becomes undetectable in most intestinal and systemic lymphoid tissues around 3 weeks post infection (PI) (Chapter 4), we postulated that this is the result of clearance of the parasite from these tissues by an immune response. Appearance of tissue cysts in visceral organs such as lungs, liver and kidneys, but predominantly in the central nervous system and in muscle tissue, has been observed 7 to 10 dpi (Black and Boothroyd, 2000). Here, they may stay for the entire host's life.

The objectives of the present study were to determine the distribution of the parasite and to assess the status of the adaptive immune response in sheep 4 to 8 weeks PI, which is shortly after the parasite left the intestinal tissues. Furthermore we wanted to compare the immune response in sheep with those in pigs. As mentioned above, humans become infected among others by ingesting tissue cysts from undercooked meat. Just like sheep, pigs are not tested for *T. gondii* infection at slaughter which makes them at risk for humans. Moreover, sheep and pigs are considered as the main source of infected meat in European countries and correlation between serological results and contamination with cysts has not been investigated (EFSA, 2007). Differences in antibody and cytokine responses against different antigens could reflect differences in host-pathogen interaction. Furthermore we aimed to obtain information on candidate antigen(s) for sero-diagnosing infection in both species.

## 5.3 Material and Methods

### 5.3.1 Parasites

*T. gondii* Prugniaud (PRU, type II) for infecting the sheep (Martrou et al., 1965) and IPB-G strain (Type II, III) for the pig study (Vercammen et al., 1998a) are routinely maintained by mouse passage. Both strains were previously used in sheep (Isabelle Moiré, personal communication) and pigs (Jongert et al., 2008; Vercammen et al., 1998a), and were harvested from the brains of chronically infected Swiss mice. Mice were euthanized by cervical dislocation, and *T. gondii* brain cysts were counted by phase-contrast microscopy. For experimental infection, the mouse brain homogenates were diluted in PBS at a concentration of 300 cysts per ml.

### 5.3.2 Animals

Four adult Belgian cross-breed sheep and six indoor-born Belgian Landrace pigs were weaned at an age of 4 weeks and housed in isolation units. The animals were *T. gondii* seronegative as determined by an indirect immunofluorescence assay (IFA). Three sheep were orally infected with 3000 tissue cysts of the *T. gondii* PRU strain and five pigs with 3000 tissue cysts of the *T. gondii* IPB-G strain. The remaining sheep and pig served as negative controls and were each given orally half a brain of a non-infected mouse.

All pigs were bled weekly from infection till euthanasia; 6 weeks PI. Sheep were bled before infection and weekly from 4 weeks PI till euthanasia, 8 weeks PI. In a previous study on sheep the antibody response had been determined on sera sampled daily from infection till 3 weeks PI (Chapter 4). Euthanasia was performed by intravenous injection of an overdose natriumpentobarbital 20% (Kela, Hoogstraten, Belgium).

Animal experiments were performed with the prior approval of the animal ethics committee of the faculties of Bioscience Engineering and Veterinary Medicine (EC 2007/103).

### **5.3.3 Detection of parasites by bio-assay and real-time quantitative PCR**

After euthanasia of the animals, 100 grams of brain, heart, *Musculus gastrocnemius* (M. gastr) and *Musculus longissimus dorsi* (M. longd) were collected from each animal for detection of parasites by bioassay and quantitative real-time PCR (qPCR). Hereto, the tissues were homogenized in 15 ml 0.9% NaCl, and the tissue suspensions were incubated 1-2 hrs in a 250 ml acidic pepsin solution ( $0.8 \text{ g l}^{-1}$  pepsin and  $7 \text{ ml l}^{-1}$  HCl) at  $37^{\circ}\text{C}$ . Thereafter, the suspension was filtered, centrifuged at 1180 g, and the pellets were resuspended in 5 ml PBS with 0.04% gentamicin. Eighty-five microliter of each suspension was used for qPCR quantification and the rest was used for the bioassay in which per tissue suspension, 5 mice were intraperitoneally inoculated with 1 ml tissue suspension each. Lungs and brains of mice that died from acute toxoplasmosis were examined for *T. gondii* parasites cysts by phase-contrast microscopy and for *T. gondii* DNA by qPCR. From surviving mice, serum was sampled at day 45 PI for testing *T. gondii* antibodies with the IFA whereafter mice were euthanized. Their brain tissues were sampled and processed for demonstrating *T. gondii* infection by qPCR as described by Kijlstra et al. (2008) and Rosenberg et al. (2009).

For qPCR, DNA was extracted with the QIAamp DNA Mini kit (Qiagen GMBH, Hilden, Germany) from 85  $\mu\text{l}$  of tissue suspension according to manufacturer's instructions. As a reference for the quantification of *T. gondii* parasites, two standard lines of 10-fold dilutions were used: one with a counted number of RH-tachyzoites and one with a counted number of cultured swine kidney cells (SK-6) as described previously (Rosenberg et al., 2009). DNA was tested by duplex Taqman-based qPCR on a BioRad iCycler (Biorad, Hercules, CA) using the *T. gondii* repeat element (AF146527) as the first target. The second target was based on the ribosomal 18s rDNA of the host cells. The reaction was performed as described by Kijlstra et al. (2008).

### **5.3.4 Indirect Immunofluorescence Antibody Test (IFA)**

The presence of IgM and IgG antibodies against *T. gondii* in sera was evaluated by the IFA. Fifty microliters of a 1/50 in PBS diluted serum sample was applied for 30 minutes at  $37^{\circ}\text{C}$  on a slide coated with formalin-treated tachyzoites from the RH strain (Toxo-Spot IF, Bio-Mérieux, Marcy-l Marcy-, France). Subsequently the slides were washed with PBS and incubated for 30 minutes at  $37^{\circ}\text{C}$  with 30  $\mu\text{l}$  of 1/50 in PBS-Evans Blue diluted fluorescein isothiocyanate (FITC) conjugated anti-sheep IgM or anti-sheep IgG, or with anti-pig IgM or



anti-pig IgG (KPL, Maryland, USA). After washing and drying, the slides were read with a fluorescence microscope (Carl Zeiss). The cut-off read-out of the fluorescence test was established with *T. gondii* seronegative and seropositive sheep and porcine reference sera 1/50 diluted in PBS. For detection of seroconversion in the mouse bio-assay, sera from these mice were tested at a 1:25 dilution and a secondary Alex 488 anti-mouse IgG antibody (Invitrogen, Merelbeke, Belgium) (1/500) was used as conjugate.

### 5.3.5 Purification of recombinant antigens

Recombinant GRA1, rGRA7 and rEC2 were purified as described previously (Bivas-Benita et al., 2003; Jongert et al., 2007; Jongert et al., 2008). The *mic3*<sub>234-307</sub> fragment was amplified from pcEC2 with forward primer 5'gcgcggtaccctccccaggatgccatt 3' and reverse primer 5' gcgcggtaccaggactggatgtcatgcc 3'. The amplicon was purified with the PCR purification kit (QIAGEN GmbH, Hilden, Germany) and digested overnight with *Bam*HI and *Hind*III, and ligated into the pQE80 expression vector (QIAGEN GmbH, Hilden, Germany). A clone was identified by colony PCR using the same primers and sequencing confirmed the presence of *mic3*<sub>234-307</sub>. Expression of rMIC3<sub>234-307</sub> was confirmed by SDS-PAGE and Western blot with serum from infected mice. The his-tagged rMIC3<sub>234-307</sub> was produced at large scale and purified according to a protocol described previously by Bivas-Benita et al. (2003).

### 5.3.6 Antibody ELISA

To measure total antigen-specific IgG antibodies, Nunc maxisorp immunoplates (Life Technologies, Ghent, Belgium) were coated for 2 hours at 37°C with TLA, rGRA1, rGRA7, rEC2 or rMIC3 at a concentration of 10  $\mu$ g/ml<sup>-1</sup> in bicarbonate coating buffer (pH 9.7).

Sheep study: In subsequent steps, the ELISA plates were blocked during 2 hours at 37°C in 1% non-fat milk powder in PBS, incubated for 1h at 37°C with 100-fold dilutions of ovine serum in PBS with 0.05% Tween® 20 and 1% non-fat milk powder and for 1 h at 37°C with rabbit anti-sheep IgG- or IgM-horseradish peroxidase (AbD Serotec, Belgium), after which a 3,3',5,5'-tetramethylbenzidine solution (TMB)(Sigma – Aldrich, Diegem, Belgium) was added. In between each step plates were manually washed 5 times with PBS 0.05% Tween® 20. The reaction was stopped by adding 50  $\mu$ l 2 N H<sub>2</sub>SO<sub>4</sub> (stop solution). Absorbance was read at 450 nm using an iMARK Microplate reader (Biorad, Nazareth,

Belgium). Positive and negative control sera were included on each plate. The cut-off value was calculated from pre-immune sera (day 0), with as cut-off value the mean  $OD_{450} + 3 \times STDEV$ . The cut-off values were: TLA: 0.409; rGRA1: 0.577; rGRA7: 0.558 rMIC3<sub>234-307</sub>: 0.4517; rEC2: 0.567

Pig study: In subsequent steps, ELISA plates were blocked overnight at 37°C in PBS 0.2% Tween®80, incubated for 1 h at 37°C with porcine serum diluted 1/50 in PBS and for 1h at 37°C with horseradish peroxidase conjugated rabbit ant-porcine gamma heavy chain antibodies (Ig; 1/1,000) (Serotec, Belgium). After this, an *o*-phenylenediamine dihydrochloride tablet (Sigma Fast; Sigma) in H<sub>2</sub>O<sub>2</sub> solution was added. In between each step, plates were washed with PBS 0.2% Tween®20. The reaction was also stopped by addition of 2 N H<sub>2</sub>SO<sub>4</sub>. Absorbance was read at 450/692 nm in an iMARK Microplate reader (Biorad, Nazareth, Belgium). Positive and negative control sera were included on each plate. The cut-off value was calculated from pre-immune sera (day 0) at a 1/50 dilution, with as cut-off value the mean  $OD_{450} + 3 \times STDEV$ . Cut-off values for ELISA were as follows: TLA: 0.095; rGRA1: 0.123; rGRA7: 0.146; rMIC3<sub>234-307</sub>: 0.136; rEC2: 0.07.

### ***5.3.7 Real-time quantitative PCR for cytokines***

Peripheral blood mononuclear cells (PBMC) were isolated by density gradient centrifugation using Lymphoprep (Nycomed, Brussels, Belgium). Ovine and porcine lymphocytes were harvested and resuspended at a concentration of  $5 \cdot 10^6$  cells ml<sup>-1</sup> in RLT buffer (Qiagen, Nazareth, Belgium) and kept at -80 °C for later isolation of cytokine mRNA. mRNA was extracted using an RNAeasy kit (Qiagen GMBH, Hilden, Germany). Reverse transcription into total cDNA was performed with the iScript kit (Biorad, Nazareth, Belgium) following the manufacturer's protocol. The obtained single-stranded cDNA was diluted 100 times for amplification in quantitative real-time PCR (qPCR). The qPCR reactions were set up in 96-well optical microtiter plates with 25 µl mixture of iQ SYBR Green Supermix (Bio-Rad, Hercules, CA). The oligonucleotide primers used for the detection of IL-4, IL-10, IFN- $\gamma$ , and the two housekeeping genes glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and Beta actin, and those for 18s rDNA are presented in Table 5.1.

**Table 5.1:** Oligonucleotide primers used in cytokine qPCR.

Cytokine	Primers (5'-3') S = sense AS= antisense
IL-10	(S) CCTGGGTTGCCAAGCCTT (AS) GCTTTGTAGACACCCCTCTCTT
IFN-gamma	(S) GAGCCAAATTGTCTCCTTCTACT (AS) CTGACTTCTCTTCCGCTTTCT
IL-4	(S) CTGGTCTGCTTACTGGYATGTA (AS) CTGTCAAGTCCGCYCAGGA
GAPDH	(S) CCATCACTGCCACCCAGAA (AS) CAGGGATGACCTTGCCCA
$\beta$ - actine	(S) GGCATCCTGACCCTCAAGTA (AS) GCCTCGGTCAGCAGCA
Ribosomal 18 S	(S) GTTGATTAAGTCCCTGCCCTTT (AS) GATAGTCAAGTTCGACCGTCTT

Each sample of cDNA was tested in duplicate, and non-template reactions were included in the runs as an internal control. For each sample, the target gene was amplified in parallel with the three house keeping control genes in separate wells. Amplification conditions were identical for all genes: a first activating cycle of the Taq polymerase of 95°C for 2 min and 45 cycles of 2 steps: 95°C for 15 sec and 60°C for 30 sec. qPCR data were analyzed using a mathematical model described by Vandesompele (2002), based on the qPCR efficiencies and the mean threshold value (Ct) deviation between the sample and control group. The normalization was done relative to the geometric average of  $\beta$ -actin, GAPDH and 18s rDNA genes, and data is represented as the normalized cytokine gene expression compared to the geometric mean number of housekeeping genes.

### 5.3.8 Statistical analysis

To determine whether an increase in antibodies or difference between the normalized quantities of cytokines post infection and these at baseline (sheep) or two weeks PI (pigs) could be observed, a Friedman two-way analysis of variance by ranks (GraphPad Prism) was performed. The same test was used for the determination of differences in the number of

parasites among the tissue samples of the animals. Differences were considered significant if the *P* value was <0.05.

## 5.4 Results

### 5.4.1 Isolation of parasites by bio-assay and detection of bradyzoites in the tissues of infected animals

Evaluation of tissue infectivity was carried out by bio-assay on tissues obtained after euthanasia of the animals.

#### Sheep study

Bio-assay and qPCR were positive for all three sheep in heart and M. gastr (Table 5.2). However, differences in results were observed for brain and M. longd. The live parasite was isolated by bio-assay from brain of all and from M. longd of one out of the three sheep, whereas parasite DNA could be detected in 2 out of the 3 brains and all 3 M. longd. The brain of one sheep, positive in bio-assay, could not be confirmed by qPCR (negative result)

**Table 5.2:** Infectivity of ovine and porcine tissues, determined by bio-assay and detection of bradyzoites by qPCR.

Tissues	Bio-assay (positive/total number)				qPCR (positive/total number)			
	Brain	Heart	M. gastr	M. longd	Brain	Hear	M. gastr	M. longd
Sheep	3/3	3/3	3/3	1/3	2/3	3/3	3/3	3/3
Pig	5/5	5/5	4/5	5/5	4/5	5/5	3/5	3/5

The control sheep remained *T. gondii* free as demonstrated by bio-assay and qPCR (data not shown).

#### Pig study

The live parasite was isolated by bio-assay from all tested tissues from all the inoculated animals six weeks after infection, except from the M.gastr of one pig (Table 5.2). These results were confirmed by qPCR for heart, but detection of parasite DNA seems to be less

sensitive since for brain, M. gastr and M. longd 1, 2 and 2 out of 5 animals, respectively, were found negative.

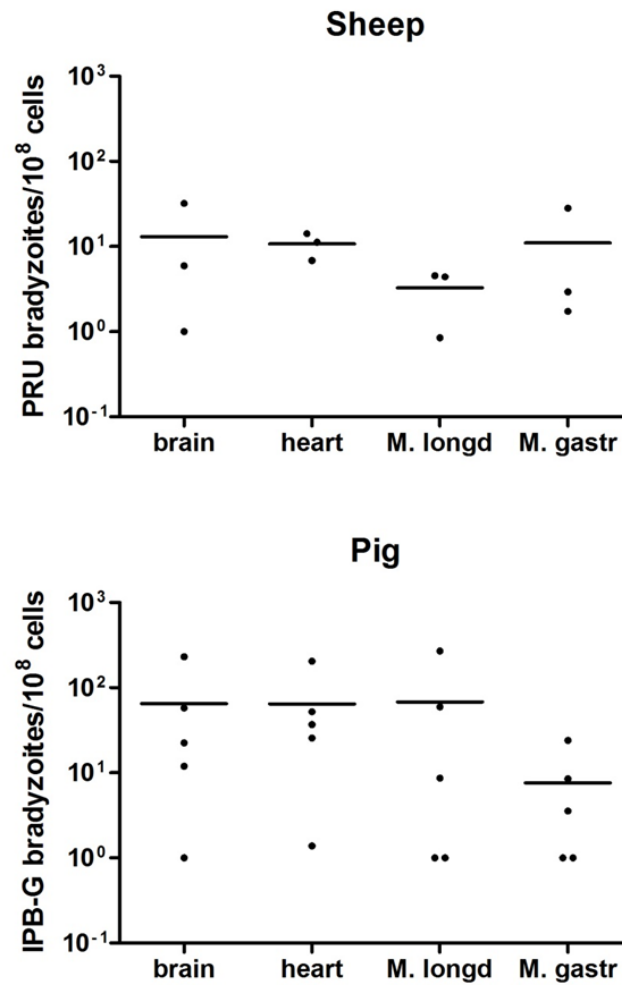
#### ***5.4.2 Quantification of the parasite load in tissues at euthanasia***

In order to determine whether parasite load was different between tissues, the numbers of bradyzoites were determined by qPCR in brain, heart, M. gastr and M. longd samples for sheep and pigs.

In sheep, brain, heart and M. gastr contained similar amounts namely  $12.76 \pm 5.74$  (SEM),  $10.78 \pm 1.24$  (SEM) and  $11.02 \pm 5.01$  (SEM) bradyzoites per  $10^8$  cells, respectively. However, M. longd contained a clearly lower amount namely  $3.27 \pm 0.70$  (SEM) bradyzoites per  $10^8$  cells (Fig. 5.1).

In pigs euthanized 6 weeks PI, the parasite load was as follows: brain:  $80.8 \pm 51.3$  (SEM), heart  $64.1 \pm 36.0$  (SEM) and M. longd  $112.7 \pm 79.9$  (SEM) bradyzoites per  $10^8$  porcine cells. In pigs the parasites were clearly less prominent in the M. gastr, with  $12.0 \pm 6.2$  (SEM) bradyzoites per  $10^8$  cells (Fig. 5.1).

The negative control animals remained *T. gondii*-free in all tissues (data not shown).



**Figure 5.1:** Bradyzoite load in tissues from sheep and pigs orally infected with *T. gondii* PRU and IPB-G respectively, determined by qPCR. The number of bradyzoites per  $10^8$  ovine/porcine cells are presented in dot plots. The horizontal lines present the mean number of parasites in each tissue at euthanasia. M. gastr = *Musculus gastrocnemius*. M. longd = *Musculus longissimus*.

### 5.4.3 Humoral response after infection

In order to evaluate kinetics of the antibody response elicited during chronic infection with *T. gondii* bradyzoites, sera were collected and tested in the Toxoplasma-specific IgM/IgG IFA and in the rGRA1, rGRA7, rMIC3<sub>234-307</sub> and rEC2-specific IgG ELISAs.

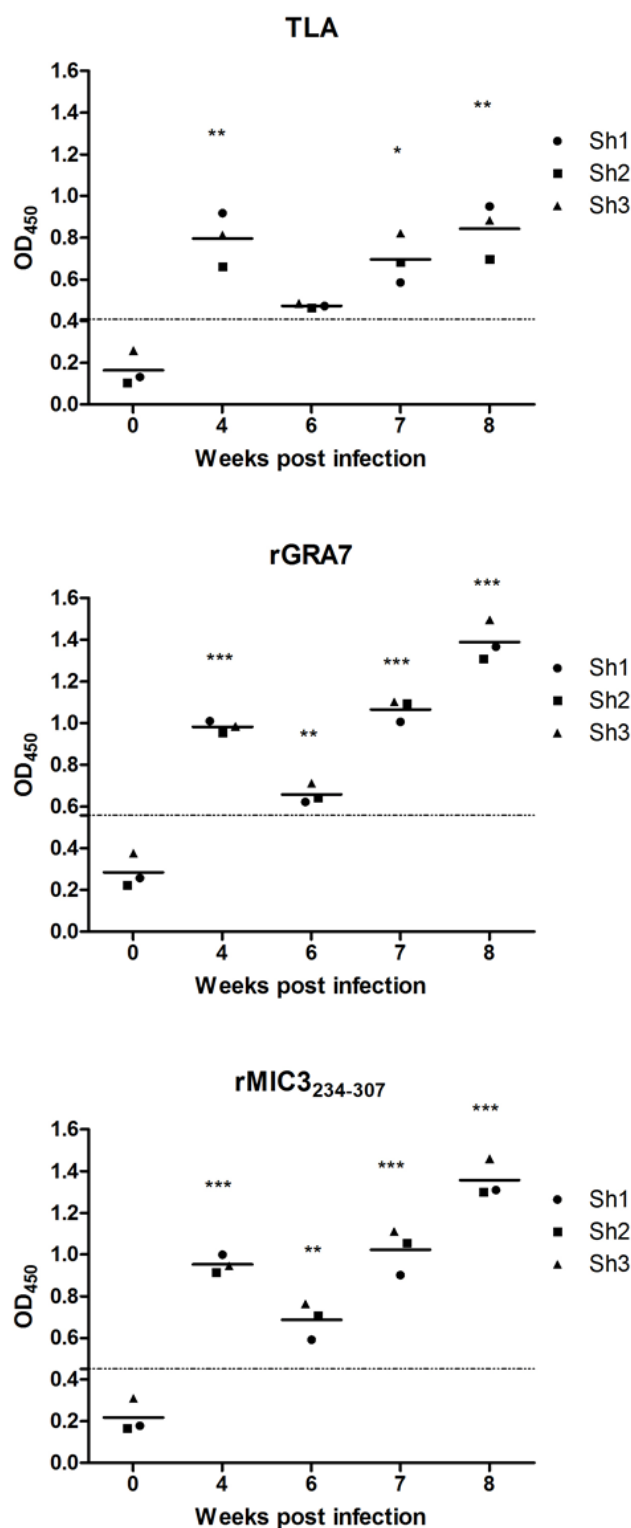
#### Sheep study

A previous study showed that most sheep become IgG positive between 1 and 3 weeks PI (Chapter 4). In the present study all three infected sheep were anti-*T. gondii* IgG seropositive in IFA 4 weeks PI. IgM was not detected between 4 and 8 weeks PI (Table 5.3).

**Table 5.3:** *Toxoplasma* seroconversion rates in IFA from sheep experimentally infected with PRU.

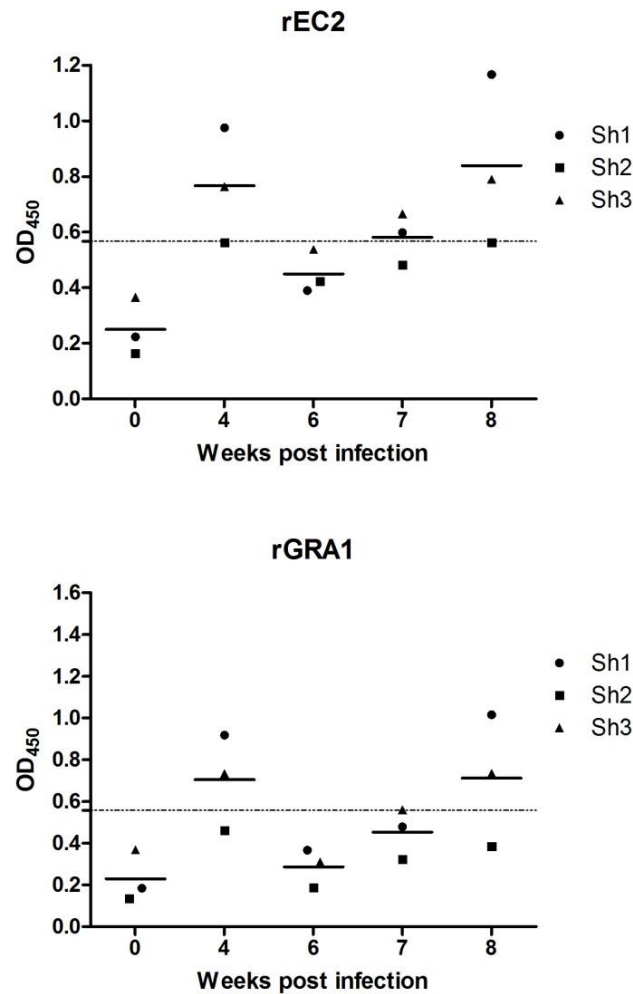
Weeks PI	0	4	5	6	7	8
IgM	0	0/3	0/3	0/3	0/3	0/3
IgG	0	3/3	3/3	3/3	3/3	3/3

In the previous study, the IgG response against *T. gondii* TLA was determined by ELISA the first 3 weeks PI (Chapter 4). In the present study, the IgG response against TLA, rGRA1, rGRA7, rEC2 or rMIC3 was studied by ELISA between 4 and 8 weeks PI (Fig. 5.2 and Fig. 5.3). The infected sheep showed similar IgG responses against TLA and the recombinant antigens. These responses were characterized by high IgG at four weeks PI, followed by a decline at 6 weeks PI, whereafter IgG increased again until euthanasia at 8 weeks PI. This was most pronounced for rGRA7 and rMIC3<sub>234-307</sub>, followed by TLA whereas the lowest responses were observed against rEC2 and rGRA1.



**Figure 5.2:** Kinetics of the antibody responses against TLA, rGRA7 and rMIC3<sub>234-307</sub> presented as OD-values for the different sheep. The lines present the mean OD-value at each time point. The dashed lines indicate the cut-off values defined with pre-immune serum. \*, \*\*, \*\*\* present significance differences at  $P < 0.05$ ,  $P < 0.01$ ,  $P < 0.001$ , respectively, in comparison with pre-immune sera





**Figure 5.3:** Kinetics of the antibody responses against rEC2 and rGRA1 presented as OD-values for the different sheep. The lines present the mean OD-value at each time point. The dashed lines indicate the cut-off values defined with pre-immune serum.

#### Pig study

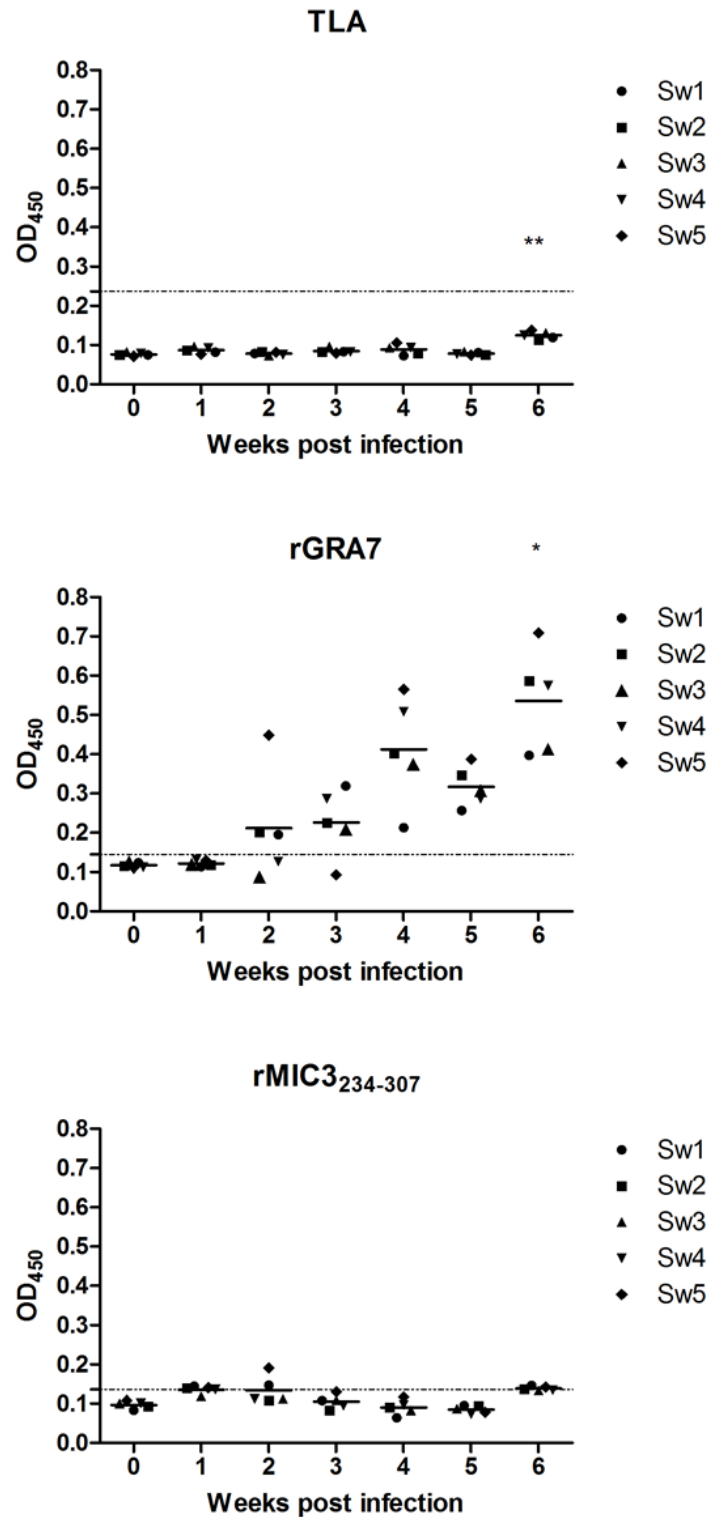
In IFA, all infected pigs were seropositive two weeks post infection (PI) for both IgG and IgM, after which a gradual decline in IgM positive animals was observed (Table 5.4). IgM disappeared by week 6 PI, while animals were still IgG positive.

**Table 5.4:** *Toxoplasma gondii* seroconversion rates in IFA from pigs experimentally infected with IPB-G

Weeks PI	0	1	2	3	4	5	6
IgM	0/5	nd	5/5	4/5	3/5	2/5	0/5
IgG	0/5	nd	5/5	3/5	4/5	5/5	5/5

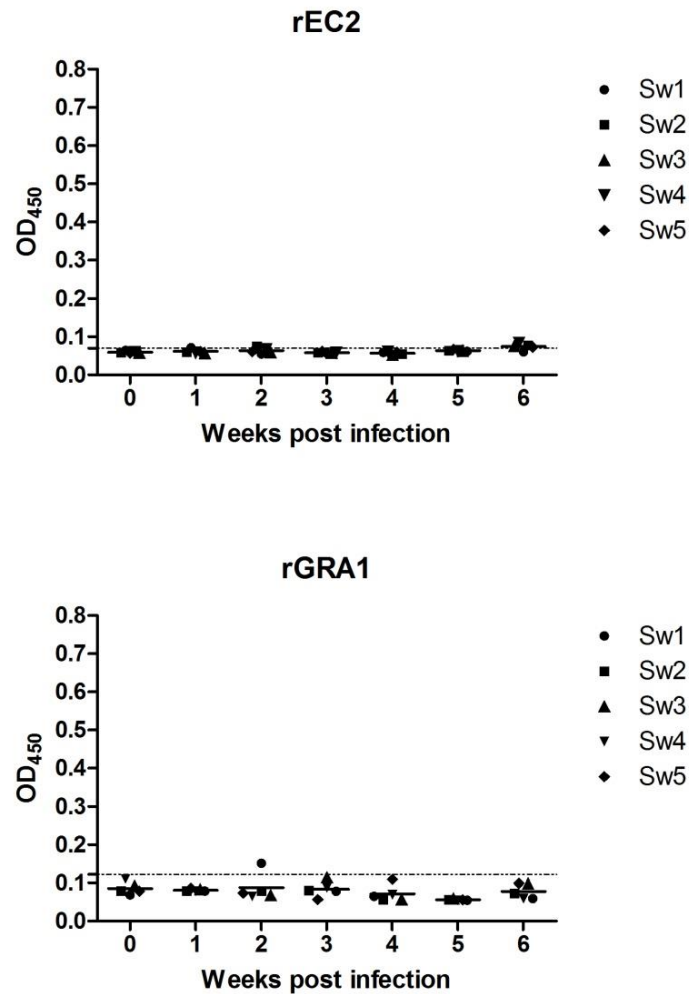
By ELISA, no or only a weak IgG response against the different recombinant antigens and TLA, was observed in pigs (Fig. 5.4) compared to sheep (Fig. 5.2), except for rGRA7. Antibodies against the dense granule protein rGRA7 appeared quickly in 3 animals as soon as 2 weeks PI. This initially moderate antibody response gradually increased so that all animals seroconverted against rGRA7 at 4 weeks PI. Interestingly, as in sheep this was followed by a moderate decline in the rGRA7-specific OD value in most pigs, whereafter the values increased again. For the other antigens either no (for TLA and rEC2) or a temporary early but very weak response was seen (for rMIC3<sub>234-307</sub> in 4 out of 5 pigs at 1 week PI and 2 out of 3 at 2 weeks PI; for rGRA1 in 1 of 5 pigs at 2 weeks PI (Fig. 5.5)). At 6 weeks PI, a tendency for increase in antibodies against most antigens, except against rGRA1, could be seen, even though values mostly remained below the cut-off.

Control pig and sheep orally infected with *T. gondii* negative brain tissue did not become seropositive in IFA or the ELISAs at any time (data not shown).



**Figure 5.4:** Kinetics of the antibody responses against TLA, rGRA7 and rMIC3<sub>234-307</sub> presented as OD-values for the different pigs.

The lines present the mean OD-value at each time point. The dashed lines indicate the cut-off values defined with pre-immune serum. \*, \*\* present significance differences at  $P < 0.05$ ,  $P < 0.01$ , respectively in comparison with pre-immune sera.



**Figure 5.5:** Kinetics of the antibody responses against rEC2 and rGRA1 presented as OD-values for the different pigs.

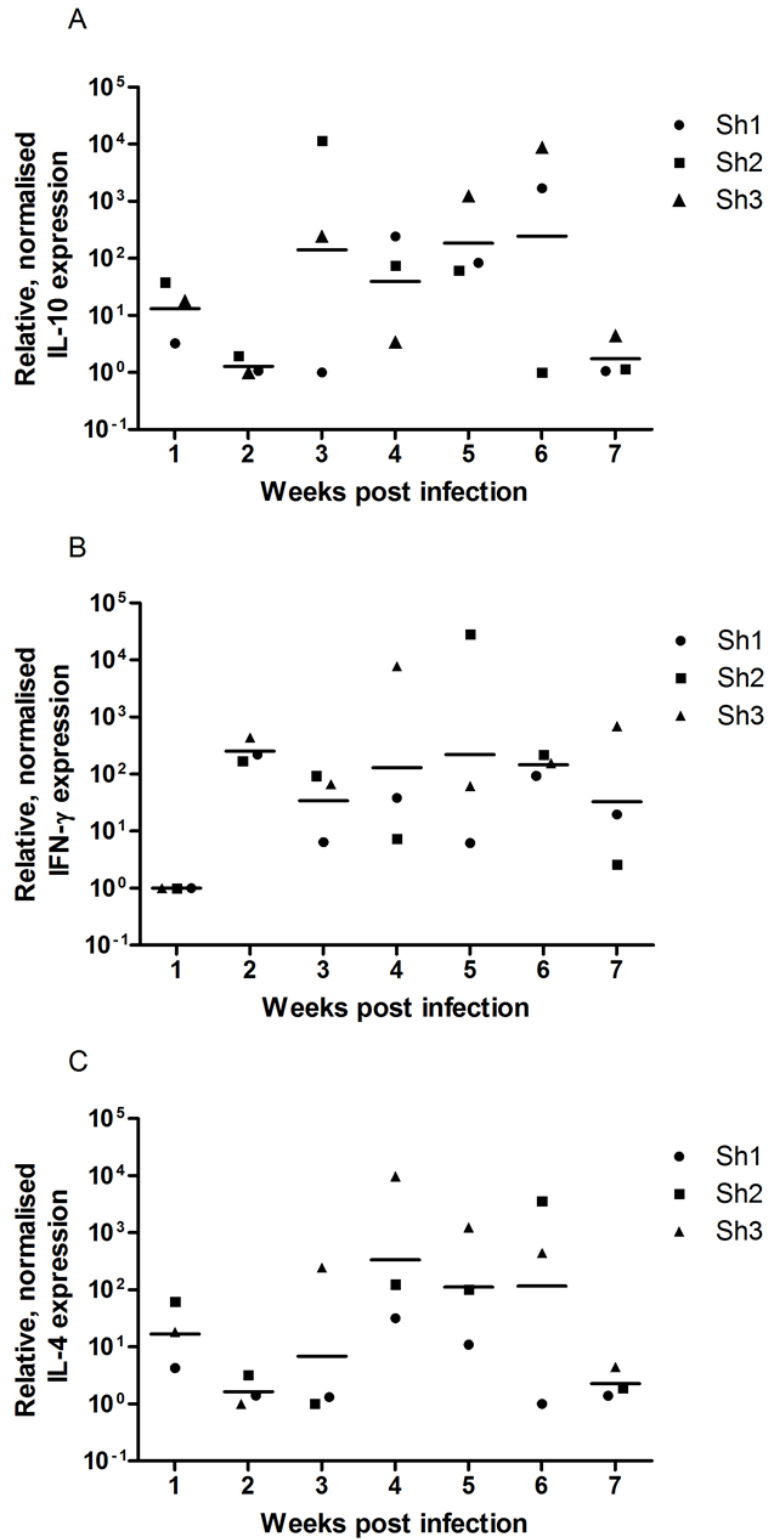
The lines present the mean OD-value at each time point. The dashed lines indicate the cut-off values defined with pre-immune serum.

#### 5.4.4 Kinetics of cytokine responses in peripheral blood mononuclear cells

In order to evaluate whether a *T. gondii* infection could elicit IFN-gamma, IL-10 and/or IL-4 in the circulating lymphocytes, PBMCs were isolated on a weekly basis until 7 weeks PI for sheep and 5 weeks PI for pigs. Relative normalized IFN-gamma, IL-4 and IL-10 mRNA levels were determined by qPCR. The cytokine levels of the control animal remained stable during the whole study period.

### Sheep study

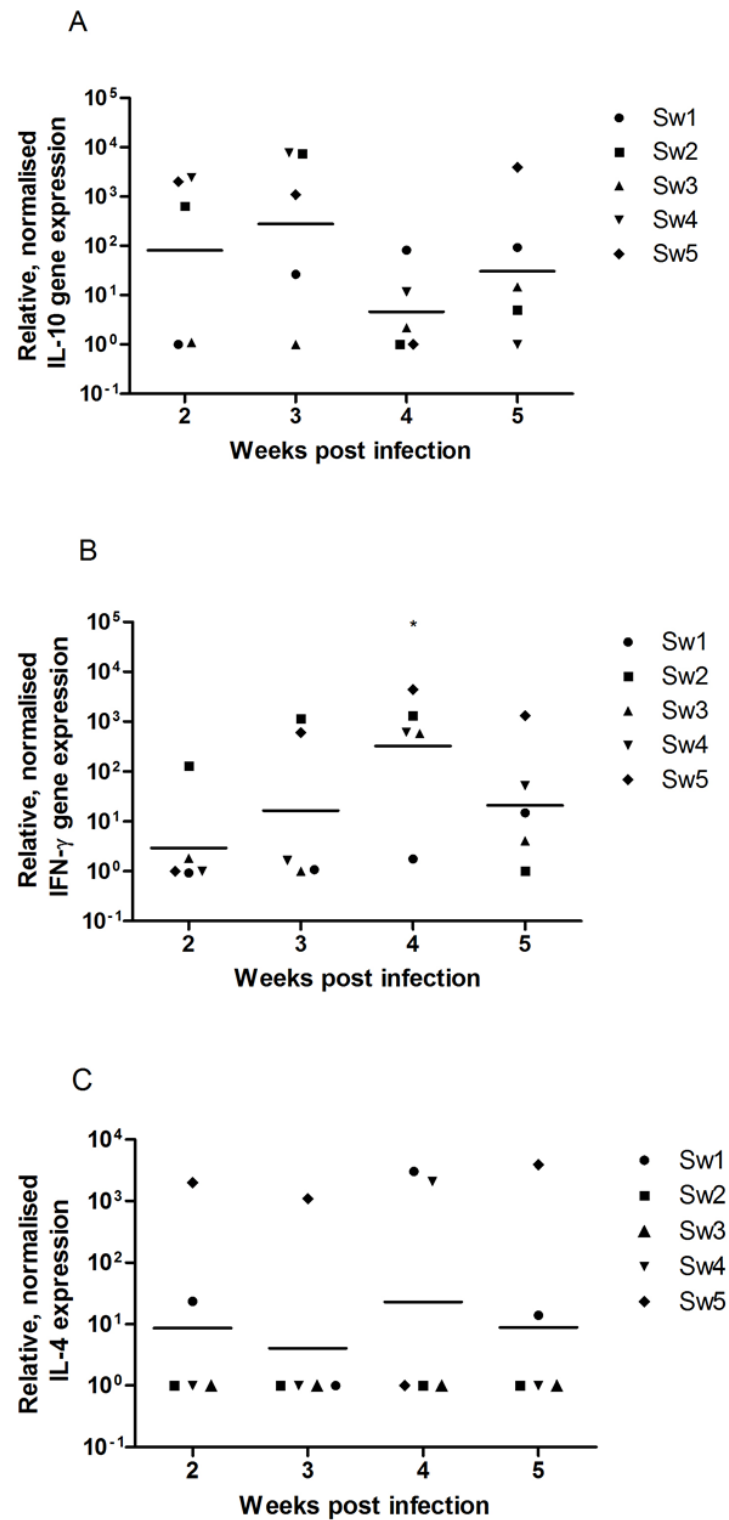
Figure 5.6 show the evolution in IL-10, IFN-gamma and IL-4 expression by PBMCs after infection of sheep. Although changes over the time after infection were not significantly different for any of the cytokines, a tendency towards increased expression could be observed. This incline was most consistent for IFN-gamma where it occurred already 2 weeks PI and persisted until the end of the observation period, even though the increase was minimal in sheep 3 at 7 weeks PI. For IL-10 and IL-4 mRNA, expression decreased in all 3 sheep at 2 weeks PI, followed by an increase between 3 and 6 weeks PI for IL-10 mRNA and between 4 and 6 weeks for IL-4 mRNA. Seven weeks PI, similar expression levels as two weeks post infection were seen (7 weeks PI).



**Figure 5.6:** Relative normalized IL-10 (A), IFN- $\gamma$  (B) and IL-4 (C) cytokine expression in PBMCs from sheep orally infected with *T. gondii* PRU are presented in scatter dot plots.

Pig study (Fig. 5.7)

In contrast to sheep, no tendency towards an increased expression was seen in pigs for IL-10 and IL-4 mRNA. IL4 mRNA even remained at basal levels in two animals throughout the observation period. IFN-gamma mRNA expression seemed to increase slightly, but later and less homogenously than in sheep, with some animals negative at all tested time points. This increase was significant at 4 weeks PI, with 4 out of 5 animals producing IFN-gamma mRNA, in comparison with 1 out of 5 IFA-seropositive animals at 2 weeks PI. Thereafter, the IFN-gamma mRNA expression declined in most animals.



**Figure 5.7:** Relative normalized IL-10 (A), IFN- $\gamma$  (B) and IL-4 (C) cytokine expression in PBMCs from pig orally infected with *T. gondii* IPB-G are presented in scatter dot plots. \* presents a significant difference at  $P < 0.05$  in comparison with 2 weeks post infection.



## 5.5 Discussion

Antibodies to *T. gondii* have been found in sheep all over the world (Dubey, 2010). The seroprevalence can be up to 90 % or even more in some countries (Dubey, 2009; Hutchinson et al., 2011) (Chapter 1). Since infected sheep remain carriers of the parasite in their tissues, mutton forms a risk for humans, especially because it is often consumed undercooked. Hence, a vaccine that could prevent infection of sheep could have a high economic value. Such a vaccine should, optimally, block infection at the site of entrance. Therefore, we previously studied the initial phase of a *T. gondii* infection and observed that the parasite disappears from intestinal tissues around 3 weeks PI (Chapter 4). Since the parasite appeared shortly after infection in blood cells and systemic lymphoid tissues, we presumed that the parasite had moved to the systemic tissues. Denkers and Gazzinelli (1998) mentioned the clearance of host tissues from the tachyzoite stage and the appearance of the bradyzoite stage in tissue cysts when the immunity builds up.

One of the aims of the present study was to demonstrate the localization of the parasite in the systemic tissues after the initial phase of infection in intestinal tissues. Furthermore, we wanted to see if the immune response and the distribution of bradyzoites in pigs were comparable to those in sheep. And thirdly, by analyzing the antibody response against different antigens, we aimed to determine the most optimal antigen to be used in an antibody ELISA.

Eight weeks after the experimental infection of sheep, parasites were detected by bioassay and qPCR in the heart, brain, M. gastrocnemius and M. longissimus dorsi of the majority of the animals. Similar amounts of parasite DNA were detected in ovine brain, heart and M. gastrocnemius, whereas the amounts were lower in the M. longissimus dorsi. This was also reflected by the results of the bioassay in which only one out of the three M. longissimus dorsi were positive. The finding of unequal distribution of the parasites in the tissues has been described previously (Denkers and Gazzinelli, 1998; Dubey et al., 1998). Bradyzoites were predominantly found in the central nervous system and heart but - depending on the host - other tissues such as liver, diaphragm and skeletal muscles are also infected (Denkers and Gazzinelli, 1998; Dubey et al., 1998). The parasite distribution in our studies suggests indeed that in sheep the parasite leaves the gut during the initial phase of infection in order to spread towards the systemic tissues (Chapter 4). In pigs, almost a similar distribution was observed, but here, the amounts of parasite DNA in M. gastrocnemius were the lowest, followed by these in the M. longissimus dorsi. Heart was consistently positive in all 8 infected animals in

qPCR as well as in bioassay, showing that the heart seems to be the most reliable tissue for demonstrating the presence of the parasite in this phase of the infection.

Additional goals of the present study were to assess the antibody and cytokine responses. In a previous study, sheep *T. gondii*-specific IgG responses against TLA were detectable from 7dpi onwards (Chapter 4). In this follow-up study, a significant IgG response was observed in sheep against TLA, rMIC3<sub>234-307</sub> and rGRA7 four weeks after the *T. gondii* infection. A similar but weaker response was observed against rEC2, a chimeric antigen encoding MIC2-MIC3-SAG1 and the weakest response against rGRA1. However, both latter responses were not statistically significant. It has been shown that TLA-specific IgM antibodies peaked three weeks PI and preceded an IgG response which remains lifelong (Dubey, 2009). *T. gondii*-specific ELISAs based on whole tachyzoites and other recombinant *T. gondii* proteins such as rSAG1, rGRA7, H4/GST, H11/GST were previously used to detect antibody responses in experimentally infected sheep (da Silva, 2002; Glor et al., 2013; Tenter et al., 1992; Velmurugan et al., 2008) or goats (Conde et al., 2001). Our study demonstrates that TLA, rMIC3<sub>234-307</sub> and rGRA7 can be used to demonstrate the infection in sheep, whereas rEC2 and rGRA1 were not reliable candidate antigens. However in pigs, rGRA7 was the only of the 5 candidate antigens against which antibodies could be demonstrated. Given that sheep were infected with a *T. gondii* type II strain and pigs with a *T. gondii* type II-III strain, we could not determine if this difference was related to the host species or to the parasite strain.

It is known that the humoral and cellular immune responses cooperate against *T. gondii* (Innes and Vermeulen, 2006; Sher et al., 1995). Sustaining strong T helper 1 (Th1)-mediated immunity, characterized by the production of IFN-gamma by CD4<sup>+</sup> and CD8<sup>+</sup> T cells, is crucial in preventing the emergence of *T. gondii* (Denkers, 1999; Gazzinelli et al., 1993). The production of IL-4 by Th2 lymphocytes is associated with disease progression and reactivation of latent disease (Hope et al., 2005). Interleukin-10 has been shown to down-regulate acute inflammatory responses against this intracellular parasite and to inhibit the IFN-gamma production (Appelberg et al., 1992; Denkers, 1999). During the acute phase of the infection (4 days PI), we studied the amounts of IFN-gamma produced in supernatants following restimulation of mononuclear cells from blood, spleen, duodenal, jejunal and ileal mesenteric lymph nodes, popliteal lymph nodes and from duodenal, jejunal and ileal Peyer's patches with different *T. gondii* antigens. We observed an early increase in IFN-gamma especially in lymphocytes of the mesenteric lymph nodes, followed by a decrease and a second increase 2 to 3 weeks PI (Chapter 4). In the follow-up study, looking at changes in mRNA expression in PBMCs directly isolated from blood, a clear increase was seen at 2

weeks PI which remained present until the last sampling 7 weeks PI. In contrast, the IL-10 and IL-4 mRNA expression did not show a consistent increase in all animals. This is in line with the Th1 response already described in sheep (Innes et al., 1995) and mice (Roberts et al., 1996). In sheep, cannulation of the efferent duct of a lymph node, draining the infection site, showed T-cells (CD4+ and/or CD8+) and IFN-gamma to be essential for host resistance during primary infection with *T. gondii* and both were seen as an important component of the protective immunity against the parasite (Innes et al., 1995). Similarly, we observed an IFN-gamma response in pigs, but this response did not seem to occur as quickly as in sheep.

In conclusion, the results in this study demonstrate that the parasite is already present in brain, heart and skeletal muscles of sheep and pigs eight and six weeks PI, respectively. The parasite was most consistently demonstrated in heart tissue with bioassay and qPCR. Interestingly, the antibody response differed considerably between sheep and pigs with significant antibody responses mainly against rGRA7, rMIC3<sub>234-307</sub> and TLA in sheep and only against rGRA7 in pigs. If this difference is species-related or due to infection with different *T. gondii* strains needs to be further studied. Nevertheless, both species showed a clear IFN-gamma response in the first weeks of infection and a seropositive response in rGRA7 ELISA. In the next studies it will be interesting to determine if this response persists in the animals and whether either methods can be confirmed to be good candidate assays for diagnosis in meat animals.

## Acknowledgements

The work described herein was funded by the institute for the Promotion of Innovation through Science and Technology” in Flanders (IWT-Vlaanderen) and the Federal Public Service, Health, Food Chain Safety and Environment, grant RF6176.

## 5.6 References

- Appelberg, R., Orme, I.M., Pinto de Sousa, M.I., Silva, M.T., 1992. In vitro effects of interleukin-4 on interferon-gamma-induced macrophage activation. *Immunology* 76, 553-559.
- Bivas-Benita, M., Laloup, M., Versteyhe, S., Dewit, J., De Braekeleer, J., Jongert, E., Borchard, G., 2003. Generation of *Toxoplasma gondii* GRA1 protein and DNA vaccine loaded chitosan particles: preparation, characterization, and preliminary in vivo studies. *Int J Pharm* 266, 17-27.
- Black, M.W., Boothroyd, J.C., 2000. Lytic cycle of *Toxoplasma gondii*. *Microbiol Mol Biol Rev* 64, 607-623.
- Conde, M., Caballero, J.M., Rodriguez-Ponce, E., Ruiz, A., Gonzalez, J., 2001. Analysis of IgG response to experimental infection with RH *Toxoplasma gondii* in goats. *Comp Immunol Microbiol Infect Dis* 24, 197-206.
- Cook, A.J., Gilbert, R.E., Buffolano, W., Zufferey, J., Petersen, E., Jenum, P.A., Foulon, W., Semprini, A.E., Dunn, D.T., 2000. Sources of *toxoplasma* infection in pregnant women: European multicentre case-control study. European Research Network on Congenital Toxoplasmosis. *BMJ* 321, 142-147.
- da Silva, A.V., Cutolo, A.A., Langoni, H., 2002. Comparação da reação de imunofluorescência indireta e do método de aglutinação direta na detecção de anticorpos anti-*toxoplasma* em soros de ovinos, caprinos, caninos e felinos. *Arq. Inst. Biol (São Paulo)* 69, 7-11.
- Denkers, E.Y., 1999. T lymphocyte-dependent effector mechanisms of immunity to *Toxoplasma gondii*. *Microbes Infect* 1, 699-708.
- Denkers, E.Y., Gazzinelli, R.T., 1998. Regulation and function of T-cell-mediated immunity during *Toxoplasma gondii* infection. *Clin Microbiol Rev* 11, 569-588.
- Dubey, J.P., 2009. Toxoplasmosis in sheep--the last 20 years. *Vet Parasitol* 163, 1-14.
- Dubey, J.P., 2010. Toxoplasmosis of Animals and Humans. (2nd ed.) CRC Press, Boca Raton, FL 33487, USA, 313 p.
- Dubey, J.P., Lindsay, D.S., Speer, C.A., 1998. Structures of *Toxoplasma gondii* tachyzoites, bradyzoites, and sporozoites and biology and development of tissue cysts. *Clin Microbiol Rev* 11, 267-299.
- EFSA, 2007. Scientific opinion of the panel on Biological Hazards and Animal Health Animal Welfare on a request from the European Food Safety Authority (self mandate) to issue a scientific opinion on the review of the Community Summary Report on Trends and Sources of Zoonoses, Zoonotic Agents and Antimicrobial Resistance in the European Union in 2005. *EFSA Journal* 1-32 (<http://www.efsa.europa.eu/en/scdocs/doc/600.pdf>).

- Gazzinelli, R.T., Denkers, E.Y., Sher, A., 1993. Host resistance to *Toxoplasma gondii*: model for studying the selective induction of cell-mediated immunity by intracellular parasites. *Infectious agents and disease* 2, 139-149.
- Glor, S.B., Edelhofer, R., Grimm, F., Deplazes, P., Basso, W., 2013. Evaluation of a commercial ELISA kit for detection of antibodies against *Toxoplasma gondii* in serum, plasma and meat juice from experimentally and naturally infected sheep. *Parasites & vectors* 6, 85.
- Hope, J.C., Kwong, L.S., Thom, M., Sopp, P., Mwangi, W., Brown, W.C., Palmer, G.H., Wattegedera, S., Entrican, G., Howard, C.J., 2005. Development of detection methods for ruminant interleukin (IL)-4. *J Immunol Methods* 301, 114-123.
- Hutchinson, J.P., Wear, A.R., Lambton, S.L., Smith, R.P., Pritchard, G.C., 2011. Survey to determine the seroprevalence of *Toxoplasma gondii* infection in British sheep flocks. *Vet Rec* 169, 582.
- Innes, E.A., Panton, W.R., Sanderson, A., Thomson, K.M., Wastling, J.M., Maley, S., Buxton, D., 1995. Induction of CD4+ and CD8+ T cell responses in efferent lymph responding to *Toxoplasma gondii* infection: analysis of phenotype and function. *Parasite Immunol* 17, 151-160.
- Innes, E.A., Vermeulen, A.N., 2006. Vaccination as a control strategy against the coccidial parasites *Eimeria*, *Toxoplasma* and *Neospora*. *Parasitology* 133 Suppl, S145-168.
- Jongert, E., de Craeye, S., Dewit, J., Huygen, K., 2007. GRA7 provides protective immunity in cocktail DNA vaccines against *Toxoplasma gondii*. *Parasite Immunol* 29, 445-453.
- Jongert, E., Melkebeek, V., De Craeye, S., Dewit, J., Verhelst, D., Cox, E., 2008. An enhanced GRA1-GRA7 cocktail DNA vaccine primes anti-*Toxoplasma* immune responses in pigs. *Vaccine* 26, 1025-1031.
- Kijlstra, A., Eissen, O.A., Cornelissen, J., Munniksmma, K., Eijck, I., Kortbeek, T., 2004. *Toxoplasma gondii* infection in animal-friendly pig production systems. *Invest Ophthalmol Vis Sci* 45, 3165-3169.
- Kijlstra, A., Jongert, E., 2008. Control of the risk of human toxoplasmosis transmitted by meat. *Int J Parasitol* 38, 1359-1370.
- Kijlstra, A., Meerburg, B., Cornelissen, J., De Craeye, S., Vereijken, P., Jongert, E., 2008. The role of rodents and shrews in the transmission of *Toxoplasma gondii* to pigs. *Vet Parasitol* 156, 183-190.
- Martrou, P., Pestre, M., Loubet, R., Nicolas, A., Malinvaud, G., 1965. La toxoplasmose congénitale (note concernant un cas mortel). *Limousin Med* 53, 3-7.
- Montoya, J.G., Liesenfeld, O., 2004. Toxoplasmosis. *Lancet* 363, 1965-1976.
- Roberts, C.W., Ferguson, D.J., Jebbari, H., Satoskar, A., Bluethmann, H., Alexander, J., 1996. Different roles for interleukin-4 during the course of *Toxoplasma gondii* infection. *Infect Immun* 64, 897-904.

- Rosenberg, C., De Craeye, S., Jongert, E., Gargano, N., Beghetto, E., Del Porto, P., Vorup-Jensen, T., Petersen, E., 2009. Induction of partial protection against infection with *Toxoplasma gondii* genotype II by DNA vaccination with recombinant chimeric tachyzoite antigens. *Vaccine* 27, 2489-2498.
- Sher, A., Denkers, E.Y., Gazzinelli, R.T., 1995. Induction and regulation of host cell-mediated immunity by *Toxoplasma gondii*. *Ciba Foundation symposium* 195, 95-104; discussion 104-109.
- Tenter, A.M., Heckeroth, A.R., Weiss, L.M., 2000. *Toxoplasma gondii*: from animals to humans. *Int J Parasitol* 30, 1217-1258.
- Tenter, A.M., Vietmeyer, C., Johnson, A.M., 1992. Development of ELISAs based on recombinant antigens for the detection of *Toxoplasma gondii*-specific antibodies in sheep and cats. *Vet Parasitol* 43, 189-201.
- Vandesompele, J., De Preter, K., Pattyn, F., Poppe, B., Van Roy, N., De Paepe, A., Speleman, F., 2002. Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. *Genome Biol* 3, RESEARCH0034.
- Velmurugan, G.V., Tewari, A.K., Rao, J.R., Baidya, S., Kumar, M.U., Mishra, A.K., 2008. High-level expression of SAG1 and GRA7 gene of *Toxoplasma gondii* (Izatnagar isolate) and their application in serodiagnosis of goat toxoplasmosis. *Vet Parasitol* 154, 185-192.
- Vercammen, M., Dardé, M.L., El Bouhdidi, A., Ben Messaoud, A., De Meuter, F., Bazin, H., Dubremetz, J.F., Carlier, Y., 1998a. Fc receptor activity of *Toxoplasma gondii* tachyzoites: 12 immunoglobulin binding by *T. gondii* strains with different isoenzyme patterns, and by SAG1 13 and SAG2 deficient mutants. In 9th International Congress of Parasitology ed. Isao Tada, 14 S.K.a.M.T., pp.1027-1032 Monduzzi Editore, Italy.

## **CHAPTER 6**

### ***GENERAL DISCUSSION***





## 6.1 Introduction

The objectives of this doctoral thesis were to generate baseline data on the seroprevalence of ovine toxoplasmosis in Belgium and to gain insight in the acute and subacute *Toxoplasma* infection in sheep. In this Chapter, I will discuss some interesting findings generated by my research and will present future prospects as well as limitations of my work.

## 6.2 Seroprevalence and risk factors for ovine toxoplasmosis in Belgium

As described in the literature review, the sexual cycle of *Toxoplasma gondii* only occurs in Felidae and results in the faecal excretion of oocysts. After sporulation, oocysts become highly infectious for all other warm-blooded animals, including humans and Felidae. In all these species, infection results in an asexual multiplication phase, which sometimes leads to clinical signs and symptoms and rarely to death, but mostly results in a latent infection with the persistence of tissue cysts in various tissues. However, when infection occurs during pregnancy, vertical transmission can occur which can lead to congenital disease and foetal mortality depending on species, immune status and stage of gestation. Ingestion of sporulated oocysts, but also ingestion of raw or undercooked meat containing *T. gondii* tissue cysts results in infection. In humans, the most important risk factor for infection is considered to be the consumption of raw or undercooked infected meat. However, water, raw vegetables and soil contaminated with oocysts may also play a role in the transmission to humans; indeed, in several studies *T. gondii* seropositive individuals have been identified among vegetarians (Dabritz and Conrad, 2010; Hall et al., 1999). Hence, waterborne as well as food-borne outbreaks have been reported from countries with diverse cultural, social and ethnic backgrounds (Bowie et al., 1997; Choi et al., 1997). About one third of the world's human population is probably latently infected (Montoya and Liesenfeld, 2004). Severe pathology as a result of *T. gondii* infection most frequently occurs in immunocompromised patients and in cases of congenital infection. The accompanying clinical effects are associated with huge economic and medical costs.

Similarly, toxoplasmosis in livestock can create economic disasters for breeders, mainly due to production losses. Ovine toxoplasmosis is widespread. The seroprevalence in sheep flocks amounts to 90% or more in some countries (Dubey, 2009). However, the infection passes often without any clinical signs. Nevertheless, based on data collected by the Veterinary Investigation Diagnosis Analysis in 2009, *T. gondii* was ranked as the second most

important abortifacient agent in the sheep industry after *Chlamydia abortus* (VIDA, 2009). The symptoms of congenital toxoplasmosis in sheep vary from early embryonic death to spontaneous abortion, mummified lambs and the birth of weak lambs with growth retardation. The economic impact due to loss of lambs has been calculated for a few countries and was estimated at about € 15.43 million per year for Great Britain (Bennett et al., 1999), € 8.62 million per year for New Zealand (Charleston, 1994), € 11.06 million for Wales (Blewett and Trees, 1987) and € 1.11-3.71 million for Uruguay (Freyre et al., 1999). *T. gondii* can thus indeed be considered as an important disease with a huge economic impact.

In Belgium very little is known about the seroprevalence of *T. gondii* in livestock. The study done in Chapter 3 showed that, at the moment of sampling, the flock prevalence in Belgium ranged from 65.2% in Antwerp to 97.8% in Flemish Brabant. A huge overall seroprevalence of 87.4% in Belgian sheep together with the identification of seropositive animals in most of the farms confirm the widespread occurrence of this parasite in sheep flocks and thus suggests an underestimation of the problem of toxoplasmosis in Belgian sheep, which could be expected since infections mostly occur subclinical and therefore mostly unnoticed. Furthermore, the methods for demonstrating the parasite in animals and their tissues (examination of tissue samples by PCR or bioassay) are too expensive and cumbersome for routine screening of sheep. Serological tests demonstrating *T. gondii*-specific antibodies (e.g. ELISA for *Toxoplasma*-antibody detection) are more feasible in routine diagnosis, but the currently used tests lack standardization, harmonization and reference material to set-up monitoring programs (EFSA, 2007), even though the EFSA Panel on Biological Hazard recommends such programs since mutton is considered to be an important source of toxoplasmosis for humans (Scorza et al., 2003).

The two possible infection routes for sheep are the vertical route from mother to foetus during pregnancy, which represents a serious risk for congenital disease (Dubey, 2009), and the horizontal route due to the ingestion of either oocysts during grazing or, tissue cysts by eating aborted tissues (placenta, foetus) (Race et al., 1998). The placenta as well as the aborted foetus can contain tissue cysts and are a risk factor for ovine toxoplasmosis (Dubey, 1987; Williams et al., 2005). This agrees with studies of Riyadh (2005) and Abu-Dalbouh et al. (2012) showing that feeding aborted foetuses to dogs can cause *T. gondii* infection and that proper disposal of foetuses on a farm decreases the risk of seroconversion of sheep with 8 percent. Eating the placenta by ewes is aimed at hiding the evidence of lambing to predators in order to protect their offspring. Nevertheless, ingestion of oocysts is considered a more

important route of transmission in sheep since lambs usually seroconvert at a very young age (unpublished observation).

The high population density in Belgium, the associated dense cat population - 26% of the households have at least one cat (Directorate-General Statistics, 2007) - the high seroprevalence of the parasite in Belgian house and stray cat populations (De Craeye et al., 2008) and the scattered distribution of farm land over many villages, are factors that are likely to cause a contamination of pastures with oocysts. Besides the human and cat population density, many other factors, among which some management factors on sheep farms, such as, insufficient hygiene measures (the non-removal of aborted tissues), no efficient cat and rodent control in the direct environment of feedstuff, surface water as the source of drinking water and farm size have proven to increase the risk of infection.

The high prevalence in sheep underlines the need for the development of more efficient diagnostic methods, which can be used to monitor the infection. Since no information exists in Belgium on the risk factors for sheep to become infected with *T. gondii*, these diagnostic methods should be used in combination with studies or questionnaires, which assess these risk factors for sheep kept under different management conditions. Furthermore, in many countries, including Belgium, vaccination is not applied, due to the disadvantages of the available commercial live vaccine such as the infection risk for the veterinarian and the limited shelf life. Therefore, risk assessment studies are crucial for the development of efficient measures to control infection and disease in our sheep population.

### **6.3 The acute and subacute immune response against *T. gondii***

In Chapter 4 and 5, respectively the acute and subacute *T. gondii* infection in sheep was studied. Many aspects such as, the parasite distribution and load, and the cellular and humoral immune responses were measured during both infection phases.

First of all we want to highlight the limitation of our studies in these chapters: (1) The numbers of animals used in our groups were very small; (2) Although we observed differences between the groups, these differences were not always statistically significant, so more data are clearly needed; (3) we were not able to determine the specificity and sensitivity of our in house ELISAs due to the lack of negative control samples. In spite of these drawbacks, we believe that we obtained some interesting results.

Host protection against *T. gondii* is complex. In mouse and sheep studies, cooperation between humoral and cellular immune mechanisms to control the infection was demonstrated

(Dubey et al., 1997; Gazzinelli et al., 1992; Sher et al., 1995). Antibodies play a role in killing extracellular tachyzoites (Hammouda et al., 1995; Kang et al., 2000), whereas cell-mediated immunity plays a major role in resistance against the parasite (Gazzinelli et al., 1991; Johnson, 1992). Parasite-specific CD8<sup>+</sup> T-cells are considered the most significant effector cells, they play a crucial role in detecting *T. gondii* (profilin) and in the subsequent induction of IL-12 production. Furthermore, they produce IFN-gamma in response to the infection, while the parasite-specific CD4<sup>+</sup> T-cells play a more synergistic role (Gaddi and Yap, 2007; Yarovsky, 2014). Natural killer (NK) cells are also believed to be important to control a *T. gondii* infection (Ge et al., 2014). Some experiments showed that *T. gondii* antigens had a direct effect on NK cells (Dannemann et al., 1989), while others supported a role for activation of accessory cells in the production of IFN-gamma by NK cells (Sher et al., 1993). However, there is no evidence that NK cells can directly suppress *T. gondii* infection. It is known that they can influence the development of an immune response through the cytokines they produce (Ge et al., 2014).

In our studies (Chapters 4 and 5), restimulating mesenteric lymph node lymphocytes with TLA, rEC2 and rMIC3 four days after infection induced high interferon-gamma responses. It has been mentioned that this early produced IFN-gamma is crucial for initiating resistance against the parasite (Bhopale, 2003; Gazzinelli et al., 1994). This IFN-gamma response only four days after infection is too early to be the result of antigen-specific CD4<sup>+</sup> and/or CD8<sup>+</sup> T lymphocyte responses. During this early stage, the parasite triggers several cells of the innate immune system, such as macrophages, natural killer cells, dendritic cells as well as neutrophils. Upon activation of these cells by *T. gondii*, some of them release cytokines such as, interleukin-12 (IL-12) and interferon-gamma (IFN-gamma) (Hunter et al., 1994a; Hunter et al., 1994b). T cell-independent IFN-gamma production by NK-cells (Sher et al., 1993) has been described in mice and is dependent on IL-12 and Tumour Necrosis Factor-alfa (TNF-alfa) production by macrophages (Gazzinelli et al., 1993). When *in vitro* purified NK cells were stimulated with *T. gondii* antigens and IL-12, they produced high levels of IFN-gamma.

Previous studies in mice have demonstrated that IL-12 production is critical to induce IFN-gamma production and protection against *T. gondii* (Vossenkamper et al., 2004). In our study, a generalised but low IL-12 production was measured in the mesenteric lymph nodes four days after infection. IL-12 rapidly induces IFN-gamma in naïve T-helper (Th) cells and promotes the development of a Th1 response, which is known from mice as well as sheep studies to be the most important response for host resistance against *T. gondii* (Buxton et al.,

2007; Innes et al., 2009; Innes and Vermeulen, 2006; Trinchieri, 2003; Trinchieri et al., 2003; Yarovinsky, 2014). In IL-12 deficient mice, *T. gondii* tachyzoites undergo uncontrolled replication, causing death (Vossenkamper et al., 2004).

We observed that rGRA7 could induce the secretion of IFN-gamma by lymphocytes isolated 10 days post infection from different mucosal and systemic lymphoid tissues, which indicates the presence of *T. gondii*-specific memory T-cells in these tissues. Moreover, two and three weeks post infection, IFN-gamma could be induced but more against TLA and/or rMIC3, indicating that GRA7 is one of the early recognized antigens and that subsequently also other antigens became recognized by T cells, broadening the immune response.

During the subacute phase of the infection (Chapter 5) high IFN-gamma mRNA levels could be detected, most likely produced by parasite-specific T lymphocytes which can prevent cyst reactivation (Denkers and Gazzinelli, 1998). The first two weeks after infection, the IFN-gamma mRNA expression by PBMCs gradually increased and subsequently remained elevated until 7 weeks post infection. This is in accordance with the strong Th1 response in sheep described by Innes et al. (1995). They concluded that CD4+ and CD8+ T-cells as well as IFN-gamma were essential for host resistance during a primary infection. IL-10 responses were also increased in all sheep, however, less consistently. Furthermore, each sheep showed a peak at a different time point between 3 and 6 weeks PI. IL-4 mRNA also increased, but slightly later than IL-10, namely between 4 and 6 weeks PI. IL-10 can be produced by many cell types, among which T regulatory lymphocytes and T-helper 2 (Th2) lymphocytes, whereas IL-4 is mainly produced by Th2 cells. Why IL-10 and IL-4 showed such a variation at different time points is not clear. Both cytokines antagonize much of the pro-inflammatory effects of interferon-gamma and inhibit the proliferation of Th1 cells, so that both cytokine responses could be seen as an attempt to control the inflammation induced by the parasitic infection. On the other hand, IFN-gamma secreted by Th1 cells blocks the proliferation of Th2 cells (Gajewski and Fitch, 1988), which could be another reason for the observed variation in production of IL-10 and IL-4. Importantly, Th2 cytokines steer the immune response towards a B cell response with antibody production. In Chapter 4 considerable variation was seen between sheep in the first appearance of *T. gondii*-specific antibodies, which could be the result of the strong early IFN-gamma response, which suppresses the Th2 responses steering the B cell response.

Interestingly, pigs did not show a clear response in IL-10 and IL-4 and the IFN-gamma response occurred slower, which could suggest a lower susceptibility of pigs to the IPB-G strain than sheep to the Prugnau strain, which resulted in a slower immune reaction. This

difference between host species could also explain the more restricted antibody response in pigs, which only clearly recognized rGRA7, compared to sheep responding against all tested antigens. Repeating our study with an experimental infection of sheep with the IPB-G-strain and pigs with the Prugniaud strain could help to answer this hypothesis.

From the induction of the cytokine gene, its transcription into a protein, its processing, trafficking and excretion, and finally the binding to its receptor and the associated induction of a response combined with many unknowns such as how many molecules will be synthesized from one mRNA and its half-life, makes it a very difficult to study protein, needing crucial planning and the use of various, performant and expensive tests.

The accurate measurement of cytokine levels is thus subject to many difficulties. Cytokines are not preformed factors but are produced as an answer on cellular activation. Then, released cytokines interact with their receptors on target cells, which are often neighbouring cells, thus only small quantities are secreted that are furthermore very short lived so that highly sensitive assays are needed for their detection (Bienvenu et al., 2000). Often sandwich ELISAs are used for measurement of cytokines, as was the case in Chapter 4 where IFN-gamma levels were measured in cell culture supernatant of *in vitro* restimulated lymphocyte or mononuclear cell cultures. This stimulation induces cytokine production by memory T cells. The measured concentrations not only depend on the cytokine secretion but also on their half-life: binding to and uptake by target cells as well as the degradation in the cell culture.

Cytokine induction can also be measured at the level of mRNA by qRT-PCR as was done in Chapter 5. The mRNA is often expressed at very low concentrations (Giulietti et al., 2001) and there is no 100% similarity between these concentrations and the actual amounts of protein secreted by the cells (Verfaillie et al., 2001).

In our study we did not identify the cells responsible for the cytokine production nor the availability of a cytokine-receptor on the target cells, which could be downregulated or even not expressed. Therefore, we are not able to define the biological activity of the measured cytokines. Different methods could be used to identify cytokine producing cells such as, cell sorting in combination with qPCR, multicolour fluorescent staining for CD markers and intracellular cytokines combined with flow cytometric analysis (Stinn et al., 1998), or immunohistochemistry, which has the advantage to identify the cytokine secreting cell types and to detect the cytokine proteins directly (Salguero et al., 2002).

In Chapter 4, we also determined the presence and distribution of the *T. gondii* parasites in the acute phase of the infection. At 4 days post infection, *T. gondii*-specific DNA was sporadically detected in enterocytes, lamina propria, mesenteric lymph nodes or Peyer's

patches and this mainly cranially in the small intestine. Four days later, the parasite seemed to have spread to more intestinal and systemic lymphoid tissues. Nevertheless, at 3 weeks post infection, the parasite seemed to disappear from most small intestinal sites, but also from popliteal lymph nodes, peripheral blood mononuclear cells and spleen. Whether this is caused by the maturation of the parasite or by the host immune response, or both is not clear. Nevertheless, at this time-point a high *T. gondii*-specific IFN-gamma memory response could be detected in the mesenteric lymph nodes, but not or less pronounced in the other tissues (Chapter 4). Five weeks later, parasites were detected by bio-assay and qPCR in heart, brain, m. gastrocnemius and m. longissimus dorsi of the majority of the sheep (Chapter 5). This distribution suggests that the parasites left the gut during the acute phase of infection to spread to the systemic tissues, where it could not be cleared by the host's immune response.

Similarly, in pigs, parasites were detected in heart, brain, m. gastrocnemius and m. longissimus dorsi. Here, qPCR was less sensitive than bio-assay to detect the parasite. A reason could be that a smaller amount of digested tissue is used in qPCR than in the bio-assay. However in sheep, this difference in sensitivity was not observed, which suggests a species-specific difference or a difference in cyst density of the inoculated strain. This finding is in agreement with previous studies in which a similar difference in sensitivity of both tests was observed in pigs (Garcia et al., 2006; Hill et al., 2006). These authors demonstrated that DNA from pig tissues interferes with the sensitivity of the *T. gondii* PCR, which is somewhat unexpected as this would rather cause more false positives than false negatives. A more plausible explanation may be that during the pepsine digestion and following DNA extraction some PCR inhibitory factors could be co-purified. It is important also to know that a negative result in PCR does not directly imply that these tissues are completely parasite free. Negative reactions can also occur due to unequal distribution of parasite cysts in the tissue samples. Dubey et al. (2010) demonstrated that the parasite load is most often higher in brain and heart or skeletal muscles than in visceral organs. In our study, the heart was consistently positive in qPCR as well as in bioassay, suggesting either a slightly higher infection load or a more equal parasite distribution in this tissue compared to others. Furthermore, heart tissue proved to be very easy to digest with pepsine, efficiently concentrating the released bradyzoites for further analyses. Therefore, the heart seems to be the most reliable tissue for demonstrating the presence of the parasite in sheep and pigs, at least this phase of the infection.

To study the kinetics of the antibody response in sheep during the acute phase of the infection we used a *T. gondii*-specific IgM and IgG IFA and also TLA- and rROP2-specific IgG ELISAs (Chapter 4). In Chapter 5, the serum IgG response of sheep and pigs against

TLA, rGRA1, rGRA7, rEC2 and rMIC3 was also tested by ELISA. It is important to stress that there is no validated or standardized serological test available for the detection of ovine toxoplasmosis. Various ELISA methods using crude, fractionated or recombinant proteins, and various antigen preparing methods are used by different labs and research groups (Chapter 2, table 2.1), which makes it difficult to compare the results.

We found that the *T. gondii*-specific IgM response measured by IFA started between 9 dpi and 15 dpi. Nearly simultaneously IgG was detected by IFA, whereas TLA-specific IgG appeared somewhat earlier, namely between 7 and 15 dpi, suggesting a slightly higher sensitivity of this ELISA test compared to the IFA. Tenter et al (1992), who infected sheep with oocysts, also found antibodies for the first time 14 days after infection. However, in two other studies in which sheep were infected with oocysts, antibody titers in IFA (Trees et al., 1989) or ELISA (Esteban-Redondo and Innes, 1997, 1998) emerged at least one week later. The slower antibody response in the latter studies is probably due to the actual infection dose: before dosing sheep with oocysts it is advisable to verify the infectivity of the inoculum by bioassay in mice as some oocysts may not be viable anymore, resulting in a lower actual inoculated infection dose. All viable stages of the parasite can infect sheep, irrespective of the dose (Dubey, 2010), but, to exclude that this difference in appearance of antibodies is due to the parasite stage used in the experimental infection, we should perform comparative infection of sheep with oocysts and tissue cysts of the same strain.

Differences in specificity and sensitivity between different serological tests will play a role in early diagnosing an antibody response: In a study on 300 ovine serum samples, four serological assays were compared: the Sabin-Feldman dye test (SFDT), the modified agglutination test (MAT), the TLA ELISA and the indirect fluorescent antibody test (IFA). The SFDT showed a sero-positivity for *T. gondii* in 34% samples, followed by IFA (37%), ELISA (41.7%) and MAT (43.7%). The IFA is highly specific but showed a low sensitivity, on the other hand the TLA ELISA demonstrated a high sensitivity, which is in accordance with our observations (Shaapan et al., 2008).

In Chapter 5, experimentally infected sheep and pigs were tested for their serum antibody response against different recombinant *T. gondii* antigens. TLA, rMIC3<sub>234-307</sub> and rGRA7 could be used to demonstrate the infection in sheep, whereas rEC2 and rGRA1 were not reliable antigens. However, in pigs, rGRA7 was the only of the 5 candidate antigens against which antibodies could be demonstrated consistently. The fact that the TLA ELISA gave poor results in pigs is somewhat unexpected as it is a crude extract prepared from *T. gondii* RH tachyzoites and should therefore contain a large repertoire of antigens. Also, since sheep and



pigs were infected with different *T. gondii* strains, we could not determine if this difference could be related to the host or to the infecting strain.

Most of homemade assays for the sero-diagnosis of *T. gondii* use the whole *Toxoplasma* soluble extract as the antigen, as we used in our ELISAs. Due to a lack of a purified standardized *Toxoplasma* antigen, or methods for the preparation of the antigen and the risk of zoonotic transmission during preparation, the availability of the use of recombinant antigens for the serological diagnosis of *T. gondii* infection would be desirable. Or alternatively, a standardized method for the preparation and use of *T. gondii* antigens should be validated and made available.

Our results of the rGRA7 ELISA were consistent with previous studies demonstrating the usefulness of *T. gondii* GRA7 as a sero-diagnostic marker of acute and chronic toxoplasmosis (Aubert et al., 2000; Bonhomme et al., 1998; Jacobs et al., 1999). Indeed, an overall sensitivity of 81 to 88% and a specificity of 98 to 100% were found for human sera in a rGRA7-specific antibody ELISA (Beghetto et al., 2006; Jacobs et al., 1999). Moreover, this ELISA had the highest positivity rates in comparison with other recombinant proteins, including the rhoptry proteins, surface antigen P30 and GRA8, another member of the dense granule proteins. Furthermore, the rGRA7 ELISA was already used to evaluate the seropositivity in pigs and showed higher positivity rates in comparison with a SAG2 and GRA14 ELISA (Terkawi et al., 2013). This difference between rGRA7 and the other recombinant proteins can be explained by the fact that the *Gra7* gene is expressed by all infectious stages of *T. gondii* and appears plentiful on the surface of the host cells, the host cell cytosol, the parasitophorous vacuolar membrane and the lumen (Holec-Gasior, 2013). After rupture of the host cells, high amounts of GRA7 come in contact with the host immune system, triggering strong antibody responses (Fischer et al., 1998).

Chimeric antigens, like rEC2, are new recombinant products. They include multiple epitopes in compound polypeptide sequences (Rosenberg et al., 2009). rEC2 is a chimeric antigen composed of MIC2, MIC3 and SAG1. Nowadays, only a few studies in humans have shown the usefulness of these chimeric recombinant proteins for the serological diagnosis of *T. gondii*. In infants born to mothers with a primary *T. gondii* infection, GST-EC2 improved the serological diagnosis (Beghetto et al., 2006). We are the first to demonstrate a clear antibody response against this chimeric antigen in sheep, but none was observed in our experimental infected pigs.

## 6.4 Conclusions

The host-parasite interface forms a delicate homeostasis between host defence and parasite survival strategies. Through our work, we aimed to provide quantitative data on the real *Toxoplasma* prevalence in sheep. Furthermore, we evaluated in this thesis whether humoral and/or cellular immunity are playing a role in this host-parasite interaction. As reported above, a high *T. gondii* seroprevalence was found in Belgian sheep. These findings draw the attention to the fact that a good risk assessment study in combination with questionnaires about farm management is indispensable for developing useful strategies to control the infection. Antibody ELISAs are applied as in-house diagnostic tests which are neither standardised nor harmonized. As a consequence, those tests are not reliable for routine surveillance in sheep as long as no validation of sensitivity and specificity has been performed. Such validation should be performed by various laboratories internationally using the same reference material.

Although the results of the present work have added new insights on the host-parasite interaction, such as the parasite distribution in the intestine and some lymphoid tissues during the acute phase of the infection and the associated antibody and cytokine responses, many elements in the immune response remain obscure. Further studies on the host-parasite interaction during the acute, subacute and even chronic infection stage might hopefully give some answers to unsolved problems such as what is triggering the IFN-gamma release in the very early phase and in the subacute phase of infection and what makes *T. gondii* disappear from the intestinal tissue.

Gaining insight into the cellular and humoral immunity during infection and the associated parasite distribution, has been the cornerstone of this thesis. During the analysis of the humoral and cellular immunity, we encountered some major obstacles: A first major obstacle for *T. gondii* infection studies in sheep was the huge seroprevalence, making it difficult to find seronegative animals for experimental infections and to obtain enough negative sera to perform the validation of our serological tests. Another important obstacle was the lack of tools: the lack of standardised serological tests and of secondary antibodies against sheep immunoglobulins and interleukins so that secondary antibodies with demonstrated cross-reactivity had to be used.

The used techniques are another focus of attention. ELISAs will assist in unravelling the role of cytokines such as IFN-gamma and other interleukins in cooperation with the antibodies against different antigens. However, in order to identify the cell types involved in

the cytokine response and to identify the antigens of the parasite responsible for a protective immune response, more sophisticated techniques have to be applied such as, multicolour flow cytometry to combine detection and quantification of cytokine production with the identification of the secreting cell type and parasite protein purification, *in vitro* proliferation and flow cytometric proliferation assays to identify parasite proteins important for stimulating the host immune cells.

Furthermore, we have to investigate if the antibody responses we found persist in the animals for a longer time and whether the rGRA7 ELISA can be confirmed to be a good assay for sero-diagnosing a *T. gondii* infection in meat producing animals throughout the acute, subacute and chronic infection stages.

## 6.5 References

- Abu-Dalbouh, M.A., Ababneh, M.M., Giadinis, N.D., Lafi, S.Q., 2012. Ovine and caprine toxoplasmosis (*Toxoplasma gondii*) in aborted animals in Jordanian goat and sheep flocks. *Trop Anim Health Prod* 44, 49-54.
- Aubert, D., Maine, G.T., Villena, I., Hunt, J.C., Howard, L., Sheu, M., Brojanac, S., Chovan, L.E., Nowlan, S.F., Pinon, J.M., 2000. Recombinant antigens to detect *Toxoplasma gondii*-specific immunoglobulin G and immunoglobulin M in human sera by enzyme immunoassay. *J Clin Microbiol* 38, 1144-1150.
- Beghetto, E., Spadoni, A., Bruno, L., Buffolano, W., Gargano, N., 2006. Chimeric antigens of *Toxoplasma gondii*: toward standardization of toxoplasmosis serodiagnosis using recombinant products. *J Clin Microbiol* 44, 2133-2140.
- Bennett, R., Christiansen, K., Clifton-Hadley, R., 1999. Preliminary estimates of the direct costs associated with endemic diseases of livestock in Great Britain. *Preventive veterinary medicine* 39, 155-171.
- Bhopale, G.M., 2003. Development of a vaccine for toxoplasmosis: current status. *Microbes Infect* 5, 457-462.
- Bienvenu, J., Monneret, G., Fabien, N., Revillard, J.P., 2000. The clinical usefulness of the measurement of cytokines. *Clin Chem Lab Med* 38, 267-285.
- Blewett, D.A., Trees, A.J., 1987. The epidemiology of ovine toxoplasmosis with especial respect to control. *Br Vet J* 143, 128-135.
- Bonhomme, A., Maine, G.T., Beorchia, A., Burlet, H., Aubert, D., Villena, I., Hunt, J., Chovan, L., Howard, L., Brojanac, S., Sheu, M., Tyner, J., Pluot, M., Pinon, J.M., 1998. Quantitative immunolocalization of a P29 protein (GRA7), a new antigen of *toxoplasma gondii*. *J Histochem Cytochem* 46, 1411-1422.
- Bowie, W.R., King, A.S., Werker, D.H., Isaac-Renton, J.L., Bell, A., Eng, S.B., Marion, S.A., 1997. Outbreak of toxoplasmosis associated with municipal drinking water. The BC *Toxoplasma* Investigation Team. *Lancet* 350, 173-177.
- Buxton, D., Maley, S.W., Wright, S.E., Rodger, S., Bartley, P., Innes, E.A., 2007. Ovine toxoplasmosis: transmission, clinical outcome and control. *Parassitologia* 49, 219-221.
- Charleston, W., 1994. *Toxoplasma* and other protozoan infections of economic importance in New Zealand. *New Zealand Journal of Zoology* 21, 67-81.
- Choi, W.Y., Nam, H.W., Kwak, N.H., Huh, W., Kim, Y.R., Kang, M.W., Cho, S.Y., Dubey, J.P., 1997. Foodborne outbreaks of human toxoplasmosis. *J Infect Dis* 175, 1280-1282.
- Dabritz, H.A., Conrad, P.A., 2010. Cats and *Toxoplasma*: implications for public health. *Zoonoses Public Health* 57, 34-52.
- Dannemann, B.R., Morris, V.A., Araujo, F.G., Remington, J.S., 1989. Assessment of human natural killer and lymphokine-activated killer cell cytotoxicity against *Toxoplasma gondii* trophozoites and brain cysts. *J Immunol* 143, 2684-2691.

- De Craeye, S., Francart, A., Chabauty, J., De Vriendt, V., Van Gucht, S., Leroux, I., Jongert, E., 2008. Prevalence of *Toxoplasma gondii* infection in Belgian house cats. *Vet Parasitol* 157, 128-132.
- Denkers, E.Y., Gazzinelli, R.T., 1998. Regulation and function of T-cell-mediated immunity during *Toxoplasma gondii* infection. *Clin Microbiol Rev* 11, 569-588.
- Directorate-General Statistics, B., 2007. De honden en katten van de Belgen. Directorate-General Statistics, Belgium, ed. (Federal Public Service Economy), 1-6.
- Dubey, J.P., 1987. *Toxoplasma gondii* cysts in placentas of experimentally infected sheep. *Am J Vet Res* 48, 352-353.
- Dubey, J.P., 2009. Toxoplasmosis in sheep--the last 20 years. *Vet Parasitol* 163, 1-14.
- Dubey, J.P., 2010. Toxoplasmosis of Animals and Humans. (2nd ed.) CRC Press, Boca Raton, FL 33487, USA, 313 p.
- Dubey, J.P., Speer, C.A., Shen, S.K., Kwok, O.C., Blixt, J.A., 1997. Oocyst-induced murine toxoplasmosis: life cycle, pathogenicity, and stage conversion in mice fed *Toxoplasma gondii* oocysts. *J Parasitol* 83, 870-882.
- EFSA, 2007. Scientific opinion of the panel on Biological Hazards and Animal Health Animal Welfare on a request from the European Food Safety Authority (self mandate) to issue a scientific opinion on the review of the Community Summary Report on Trends and Sources of Zoonoses, Zoonotic Agents and Antimicrobial Resistance in the European Union in 2005. *EFSA Journal* 1-32 (<http://www.efsa.europa.eu/en/scdocs/doc/600.pdf>).
- Esteban-Redondo, I., Innes, E.A., 1997. *Toxoplasma gondii* infection in sheep and cattle. *Comp Immunol Microbiol Infect Dis* 20, 191-196.
- Esteban-Redondo, I., Innes, E.A., 1998. Detection of *Toxoplasma gondii* in tissues of sheep orally challenged with different doses of oocysts. *Int J Parasitol* 28, 1459-1466.
- Fischer, H.G., Stachelhaus, S., Sahm, M., Meyer, H.E., Reichmann, G., 1998. GRA7, an excretory 29 kDa *Toxoplasma gondii* dense granule antigen released by infected host cells. *Mol Biochem Parasitol* 91, 251-262.
- Freyre, A., Bonino, J., Falcon, J., Castells, D., Correa, O., Casaretto, A., 1999. The incidence and economic significance of ovine toxoplasmosis in Uruguay. *Vet Parasitol* 81, 85-88.
- Gaddi, P.J., Yap, G.S., 2007. Cytokine regulation of immunopathology in toxoplasmosis. *Immunology and cell biology* 85, 155-159.
- Gajewski, T.F., Fitch, F.W., 1988. Anti-proliferative effect of IFN-gamma in immune regulation. I. IFN-gamma inhibits the proliferation of Th2 but not Th1 murine helper T lymphocyte clones. *J Immunol* 140, 4245-4252.
- Garcia, J.L., Gennari, S.M., Machado, R.Z., Navarro, I.T., 2006. *Toxoplasma gondii*: detection by mouse bioassay, histopathology, and polymerase chain reaction in tissues from experimentally infected pigs. *Exp Parasitol* 113, 267-271.
- Gazzinelli, R., Xu, Y., Hieny, S., Cheever, A., Sher, A., 1992. Simultaneous depletion of CD4+ and CD8+ T lymphocytes is required to reactivate chronic infection with *Toxoplasma gondii*. *J Immunol* 149, 175-180.

- Gazzinelli, R.T., Denkers, E.Y., Sher, A., 1993. Host resistance to *Toxoplasma gondii*: model for studying the selective induction of cell-mediated immunity by intracellular parasites. *Infectious agents and disease* 2, 139-149.
- Gazzinelli, R.T., Hakim, F.T., Hieny, S., Shearer, G.M., Sher, A., 1991. Synergistic role of CD4+ and CD8+ T lymphocytes in IFN-gamma production and protective immunity induced by an attenuated *Toxoplasma gondii* vaccine. *J Immunol* 146, 286-292.
- Gazzinelli, R.T., Wysocka, M., Hayashi, S., Denkers, E.Y., Hieny, S., Caspar, P., Trinchieri, G., Sher, A., 1994. Parasite-induced IL-12 stimulates early IFN-gamma synthesis and resistance during acute infection with *Toxoplasma gondii*. *J Immunol* 153, 2533-2543.
- Ge, Y., Chen, J., Qiu, X., Zhang, J., Cui, L., Qi, Y., Liu, X., Qiu, J., Shi, Z., Lun, Z., Shen, J., Wang, Y., 2014. Natural killer cell intrinsic toll-like receptor MyD88 signaling contributes to IL-12-dependent IFN-gamma production by mice during infection with *Toxoplasma gondii*. *Int J Parasitol* 44, 475-484.
- Giulietti, A., Overbergh, L., Valckx, D., Decallonne, B., Bouillon, R., Mathieu, C., 2001. An overview of real-time quantitative PCR: applications to quantify cytokine gene expression. *Methods* 25, 386-401.
- Hall, S.M., Pandit, A., Golwilkar, A., Williams, T.S., 1999. How do Jains get *toxoplasma* infection? *Lancet* 354, 486-487.
- Hammouda, N.A., Abo el-Naga, I., Hussein, E.D., Rashwan, E.A., 1995. Opsonization and intracellular killing of *Toxoplasma gondii* by human mononuclear phagocytes. *Journal of the Egyptian Society of Parasitology* 25, 11-17.
- Hill, D.E., Chirukandoth, S., Dubey, J.P., Lunney, J.K., Gamble, H.R., 2006. Comparison of detection methods for *Toxoplasma gondii* in naturally and experimentally infected swine. *Vet Parasitol* 141, 9-17.
- Holec-Gasior, L., 2013. *Toxoplasma gondii* recombinant antigens as tools for serodiagnosis of human toxoplasmosis: current status of studies. *Clin Vaccine Immunol* 20, 1343-1351.
- Hunter, C.A., Subauste, C.S., Remington, J.S., 1994a. The role of cytokines in toxoplasmosis. *Biotherapy* 7, 237-247.
- Hunter, C.A., Subauste, C.S., Van Cleave, V.H., Remington, J.S., 1994b. Production of gamma interferon by natural killer cells from *Toxoplasma gondii*-infected SCID mice: regulation by interleukin-10, interleukin-12, and tumor necrosis factor alpha. *Infect Immun* 62, 2818-2824.
- Innes, E.A., Bartley, P.M., Buxton, D., Katzer, F., 2009. Ovine toxoplasmosis. *Parasitology* 136, 1887-1894.
- Innes, E.A., Panton, W.R., Sanderson, A., Thomson, K.M., Wastling, J.M., Maley, S., Buxton, D., 1995. Induction of CD4+ and CD8+ T cell responses in efferent lymph responding to *Toxoplasma gondii* infection: analysis of phenotype and function. *Parasite Immunol* 17, 151-160.
- Innes, E.A., Vermeulen, A.N., 2006. Vaccination as a control strategy against the coccidial parasites *Eimeria*, *Toxoplasma* and *Neospora*. *Parasitology* 133 Suppl, S145-168.
- Jacobs, D., Vercammen, M., Saman, E., 1999. Evaluation of recombinant dense granule antigen 7 (GRA7) of *Toxoplasma gondii* for detection of immunoglobulin G antibodies and analysis of a major antigenic domain. *Clin Diagn Lab Immunol* 6, 24-29.

- Johnson, L.L., 1992. SCID mouse models of acute and relapsing chronic *Toxoplasma gondii* infections. *Infect Immun* 60, 3719-3724.
- Kang, H., Remington, J.S., Suzuki, Y., 2000. Decreased resistance of B cell-deficient mice to infection with *Toxoplasma gondii* despite unimpaired expression of IFN-gamma, TNF-alpha, and inducible nitric oxide synthase. *J Immunol* 164, 2629-2634.
- Montoya, J.G., Liesenfeld, O., 2004. Toxoplasmosis. *Lancet* 363, 1965-1976.
- Race, R., Jenny, A., Sutton, D., 1998. Scrapie infectivity and proteinase K-resistant prion protein in sheep placenta, brain, spleen, and lymph node: implications for transmission and antemortem diagnosis. *J Infect Dis* 178, 949-953.
- Riyadh, R., 2005. Flock level sero-prevalence of and risk factors for *Toxoplasma gondii* in sheep and goats in northern Jordan. unpublished Master's thesis, Jordan University of Science and Technology, Irbid, Jordan.
- Rosenberg, C., De Craeye, S., Jongert, E., Gargano, N., Beghetto, E., Del Porto, P., Vorup-Jensen, T., Petersen, E., 2009. Induction of partial protection against infection with *Toxoplasma gondii* genotype II by DNA vaccination with recombinant chimeric tachyzoite antigens. *Vaccine* 27, 2489-2498.
- Salguero, F.J., Ruiz-Villamor, E., Bautista, M.J., Sanchez-Cordon, P.J., Carrasco, L., Gomez-Villamandos, J.C., 2002. Changes in macrophages in spleen and lymph nodes during acute African swine fever: expression of cytokines. *Vet Immunol Immunopathol* 90, 11-22.
- Scorza, T., D'Souza, S., Laloup, M., Dewit, J., De Braekeleer, J., Verschueren, H., Vercammen, M., Huygen, K., Jongert, E., 2003. A GRA1 DNA vaccine primes cytolytic CD8(+) T cells to control acute *Toxoplasma gondii* infection. *Infect Immun* 71, 309-316.
- Shaapan, R.M., El-Nawawi, F.A., Tawfik, M.A.A., 2008. Sensitivity and specificity of various serological tests for the detection of *Toxoplasma gondii* infection in naturally infected sheep. *Vet. Parasitol.* 153, 359-362.
- Sher, A., Denkers, E.Y., Gazzinelli, R.T., 1995. Induction and regulation of host cell-mediated immunity by *Toxoplasma gondii*. *Ciba Foundation symposium* 195, 95-104; discussion 104-109.
- Sher, A., Oswald, I.P., Hieny, S., Gazzinelli, R.T., 1993. *Toxoplasma gondii* induces a T-independent IFN-gamma response in natural killer cells that requires both adherent accessory cells and tumor necrosis factor-alpha. *J Immunol* 150, 3982-3989.
- Stinn, J.L., Taylor, M.K., Becker, G., Nagano, H., Hasegawa, S., Furakawa, Y., Shimizu, K., Libby, P., Mitchell, R.N., 1998. Interferon-gamma-secreting T-cell populations in rejecting murine cardiac allografts: assessment by flow cytometry. *Am J Pathol* 153, 1383-1392.
- Tenter, A.M., Vietmeyer, C., Johnson, A.M., 1992. Development of ELISAs based on recombinant antigens for the detection of *Toxoplasma gondii*-specific antibodies in sheep and cats. *Vet Parasitol* 43, 189-201.
- Terkawi, M.A., Kameyama, K., Rasul, N.H., Xuan, X., Nishikawa, Y., 2013. Development of an immunochromatographic assay based on dense granule protein 7 for serological detection of *Toxoplasma gondii* infection. *Clin Vaccine Immunol* 20, 596-601.

- Trees, A.J., Crozier, S.J., Buxton, D., Blewett, D.A., 1989. Serodiagnosis of ovine toxoplasmosis: an assessment of the latex agglutination test and the value of IgM specific titres after experimental oocyst-induced infections. *Res Vet Sci* 46, 67-72.
- Trinchieri, G., 2003. Interleukin-12 and the regulation of innate resistance and adaptive immunity. *Nature reviews. Immunology* 3, 133-146.
- Trinchieri, G., Pflanz, S., Kastelein, R.A., 2003. The IL-12 family of heterodimeric cytokines: new players in the regulation of T cell responses. *Immunity* 19, 641-644.
- Verfaillie, T., Cox, E., To, L.T., Vanrompay, D., Bouchaut, H., Buys, N., Goddeeris, B.M., 2001. Comparative analysis of porcine cytokine production by mRNA and protein detection. *Vet Immunol Immunopathol* 81, 97-112.
- VIDA, 2009. Veterinary Investigation Surveillance Report
- Vossenkamper, A., Struck, D., Alvarado-Esquivel, C., Went, T., Takeda, K., Akira, S., Pfeffer, K., Alber, G., Lochner, M., Forster, I., Liesenfeld, O., 2004. Both IL-12 and IL-18 contribute to small intestinal Th1-type immunopathology following oral infection with *Toxoplasma gondii*, but IL-12 is dominant over IL-18 in parasite control. *Eur J Immunol* 34, 3197-3207.
- Williams, R.H., Morley, E.K., Hughes, J.M., Duncanson, P., Terry, R.S., Smith, J.E., Hide, G., 2005. High levels of congenital transmission of *Toxoplasma gondii* in longitudinal and cross-sectional studies on sheep farms provides evidence of vertical transmission in ovine hosts. *Parasitology* 130, 301-307.
- Yarovinsky, F., 2014. Innate immunity to *Toxoplasma gondii* infection. *Nature reviews. Immunology* 14, 109-121.



## **SUMMARY**



According to The Scientific Opinion of the Panel on Biological Hazards, toxoplasmosis has the highest human incidence amongst the parasitic zoonoses. Toxoplasmosis is considered to be an under-detected and underreported disease in the European Union. Epidemiological data and case-control reports identify un- or undercooked meat as an important source of human toxoplasmosis. The same panel highlighted the limitation of quantitative data on toxoplasmosis contamination levels in Belgian sheep flocks as an important information gap in Belgium, as well as in some other countries. Aside from the gap in quantitative data, significant gaps still exist in our knowledge on the host-immune response during acute and chronic *T. gondii* infection.

In the **first two chapters** an overview was given of the life cycle, pathogenesis, economic aspects and diagnostic tools of *T. gondii*, with specific focus on sheep.

The general aims of this PhD thesis were a) to provide data on the prevalence of *Toxoplasma gondii* in Belgian sheep flocks, and b) to have a better insight in the parasite-host interaction, with specific focus on the correlation between the host-immune response on one hand and the parasite distribution on the other hand.

In **Chapter 3**, we provide the first data on the prevalence of a *T. gondii* infection in Belgian sheep flocks. A total of 3170 serum samples were tested for antibodies against *T. gondii* in a total parasitic lysate (TLA)-specific antibody enzyme-linked immunosorbent assay (ELISA). Almost 2400 (87.4%) out of the 3170 samples were positive for *T. gondii*. The province of Antwerp showed the lowest seroprevalence (65.2%), followed by the Walloon region (68.6%), East Flanders (96.6%), West Flanders (96.8%) and Limburg (97.3%). Flemish Brabant noted the highest seroprevalence of 97.8%. Only 8 of the 209 tested flocks were seronegative. The study revealed a significantly lower *T. gondii* seroprevalence in Antwerp and Wallonia than in the other Flemish provinces ( $p < 0.05$ ). The overall high prevalence in Belgium demonstrates that more attention has to be given to mutton as a potential source of human toxoplasmosis.

In **Chapter 4**, the parasite distribution and immune response, following oral ingestion by sheep of tissue cysts of the *T. gondii* PRU strain was studied during the acute phase of the infection. The presence of parasite DNA as well as parasite-induced interferon-gamma and interleukine-12 responses were determined at 4, 8, 10, 14 and 21 days post infection in the small intestinal mucosa, draining mesenteric lymph nodes, popliteal LN, spleen and blood mononuclear cells. Furthermore, the level of *T. gondii*-specific serum antibodies was tested

daily by indirect immunofluorescence assay for IgM and IgG and by a total parasitic lysate-specific ELISAs for IgG, IgG1, IgG2 and IgA. Results indicate that tachyzoites already enter the cranial small intestinal mucosa the first days after infection while the sheep are still seronegative. Simultaneously, high IFN-gamma responses and IL-12 are induced mainly in mesenteric lymph nodes. IgG1, first seen at 8dpi, and IgG2 at 11 dpi, are accompanied with a decrease or even disappearance of the IFN-gamma and IL-12 response in PP, PBMC and popliteal LN. Meanwhile the parasite DNA could be recovered from most mucosal and systemic tissues to become undetectable in the small intestine, popliteal LN, PBMC and spleen 3 weeks after infection, suggesting that the parasite is leaving these tissues. Our results suggest a role for both cytokines in controlling the infection.

In **Chapter 5** we performed a follow up study to see how the IFN-gamma (T-helper 1 cells), IL-4 (Th2 cells) and IL-10 mRNA (Treg cells) expression by blood mononuclear cells, and the serum antibody response against *T. gondii* total lysate antigen, recombinant GRA1, rGRA7, rMIC3 and rEC2, evolved in sheep the first two months after a *T. gondii* infection. Furthermore, we compared these responses with the responses in pigs to see if important species differences occurred. At the end of this period, the parasite distribution, in heart, brain and two skeletal muscles in sheep was compared with that in pigs, with the aim to relate differences in immune responses between both species with differences in parasite load. Both antibody and cytokine responses differed between the two species. In sheep, antibodies appeared against all tested *T. gondii* antigens, but mainly against rGRA7, rMIC3 and TLA, whereas in pigs only rGRA7-specific antibodies could be demonstrated. Similarly, the cytokine response seemed to be more pronounced in sheep than in pigs. In all sheep an IFN-gamma response occurred already two weeks post infection and persisted throughout the seven week observation period, whereas in pigs IFN-gamma increased later and seemingly for a shorter period. Furthermore, also IL-10 and IL-4 increased in sheep, but later than IFN-gamma and with more variation. However, in pigs no such increase was seen. These differences in immune response between sheep and pigs did not seem to affect the parasite distribution, since it was similar in both species 6 to 8 weeks post infection. Interestingly, only heart tissue was consistently positive in sheep and pigs using either bioassay or qPCR.

Results of our study therefore suggest that serum antibodies against GRA7 in live sheep and pigs and heart tissue for bioassay and qPCR in slaughtered animals seem to be excellent ways to diagnose the presence of a *T. gondii* infection between one and two months post infection. It still needs to be examined if this is also the case in chronically infected animals.

**Chapter 6** represents the general discussion and conclusions with respect to the obtained results. In this thesis, for the first time the seroprevalence of *T. gondii* in the Belgian sheep population was measured. Nevertheless, critical considerations can be made concerning the assays used for testing sheep serum. The IFA used is not validated and standardized for sheep and the TLA-specific ELISA we used, is an in house made test and needs further evaluation to be used as a screening test for identifying infected animals. Furthermore, the high seroprevalence of *T. gondii* in sheep stresses an urgent need for risk assessment studies on Belgian sheep farms with different management systems.

Additionally, we demonstrated that, during an acute infection, high interferon-gamma responses occurred, first most likely as a result of NK cell activation but later via Th1 response. At 3 weeks post infection the parasite disappeared from intestinal tissues. Even though *T. gondii*-specific serum antibody concentrations and the Th1 cytokine response increased, it is unclear if the immune response is responsible for the parasites disappearing from the small intestinal and systemic lymphoid tissues or if the parasite lost tropism for gut cells. Nevertheless, the parasite could be detected in brain, heart and skeletal muscles around two months post infection showing that the immune response could not clear the parasite from these tissues.

Some important remarks have to be taken into account when interpreting the immune response. First of all, the amount of cytokines measured in the cell culture supernatant by ELISA does not always correspond with the cytokine mRNA level detected by a real-time polymerase chain reaction. Furthermore it does not identify the cells responsible for the cytokine production. Identifying the cells responsible for the cytokine production in different phases of the infection is needed to better understand the host-parasite interaction. Secondly it is important to know that a negative result in PCR does not directly imply that these tissues are completely parasite free. One of the reasons for false negative PCR results is that only small samples are tested and that the parasite can be unequally distributed over a tissue. Thirdly, it is important to stress that there are no validated or standardized serological tests available in sheep to sero-diagnose the infection. Fourthly, since sheep and pigs were infected with different type II *T. gondii* strains, we could not determine if the observed differences were related to the host or to the *T. gondii* strain.

This thesis was a first step towards new insights in *T. gondii* infections in sheep in Belgium, in the host-parasite interaction in the small intestine during the acute phase of the infection and of antibody and cytokine responses during the first months of infection in relation to parasite distribution. However, there is much yet to be done, such as identifying risk factors of infection on Belgian sheep farms, identifying the immune cells responsible for the cytokine responses and perhaps for clearing the parasite from intestinal tissues during acute *T. gondii* infection in sheep and improving and standardizing tests to detect *T. gondii* in live and slaughtered animals.

## **SAMENVATTING**





Volgens de Scientific Opinion of the Panel on Biological Hazards heeft toxoplasmosis de hoogste humane incidentie van de parasitaire zoönosen. Desalniettemin wordt toxoplasmose beschouwd als een ondergediagnosticeerde en -gerapporteerde ziekte binnen de Europese unie. Epidemiologische data en case-control studies aanzien de inname van rauw of onvoldoende doorbakken vlees als een belangrijke oorzaak van humane toxoplasmosis. Risico-analyses, uitgevoerd door hetzelfde panel, duiden het gebrek aan van kwantitatieve data op Belgisch niveau.

Naast dit gebrek aan epidemiologische gegevens in België, is er ook nog steeds weinig gekend over de immuunrespons bij het schaap tijdens een *T. gondii* infectie. De doelstellingen van deze doctoraatsthesis waren a) het bekomen van basisgegevens over de seroprevalentie in de Belgische schapenhouderij b) het verkrijgen van een beter inzicht in de parasiet – gastheer interactie, met focus op de correlatie tussen de immuunrespons van de gastheer enerzijds en de parasitaire distributie anderzijds.

Het proefschrift bestaat uit twee grote delen, een literatuuroverzicht en een beschrijving van de experimentele studies.

In de **literatuurstudie** wordt in een eerste hoofdstuk een algemeen overzicht gegeven van *Toxoplasma gondii*, met speciale aandacht voor de levenscyclus. Vervolgens wordt in een tweede hoofdstuk de nadruk gelegd op *T. gondii* infecties bij schapen. Hierin wordt een orale infectie door oöcysten uit de omgeving, en de verticale overdracht door de parasiet samen met de pathogenese besproken. Daarna wordt een overzicht gegeven van de seroprevalentie van *T. gondii* bij schapen in verschillende landen. Dit toont aan dat toxoplasmosis wereldwijd voorkomt bij schapen waar het één van de belangrijkste oorzaken is van abortus. Hierdoor heeft deze infectie een grote economische impact op de schapenhouderij. Vervolgens wordt de aard van de immuunrespons beschreven waarbij de cruciale rol van celgemedieerde immuniteit in het controleren van de infectie wordt benadrukt. De meeste data over de immuunrespons tegenover *T. gondii* zijn afkomstig van studies in muismodellen en moeten bij schapen nog aangetoond worden. Bij de preventieve maatregelen om infectie te voorkomen worden naast vaccinatie en medicatie, het in acht nemen van bedrijfshygiëne en aanpassingen in het bedrijfsmanagement behandeld. Ofschoon een commercieel geattenuëerd vaccin beschikbaar is voor gebruik bij schapen, wordt dit in veel landen - waaronder België - niet toegelaten omdat het gebruik gepaard gaat met risico's op infectie voor de dierenarts. Bovendien biedt dit vaccin wel bescherming tegen spontane abortus tijdens de dracht maar biedt het geen steriele immuniteit voor de foetus noch voor het moederdier, waardoor

onderzoek naar vaccinatie nog steeds nodig is. Profylactische behandeling van ooien tijdens de dracht is effectief maar duur. Tenslotte wordt in dit hoofdstuk dieper ingegaan op de verschillende diagnostische detectiemethoden. Het belang van de verschillende serodiagnostische testen, met elk hun eigen voor- en nadelen, wordt uiteengezet. Daarnaast worden de technieken voor het aantonen van parasitair DNA besproken. Methodes voor de directe detectie van *T. gondii* zijn meestal technisch moeilijker en worden in veterinaire diagnostiek bijna uitsluitend gebruikt ter bevestiging van een *Toxoplasma* infectie in geaborteerde foeti of dode dieren. Het gebrek aan een gevalideerde en gestandaardiseerde diagnostische test wordt in het laatste deel belicht.

In **hoofdstuk 3** worden de resultaten van de *Toxoplasma* seroprevalentie studie op Belgische schapenbedrijven besproken. Deze studie werd uitgevoerd in samenwerking met de Dierengezondheidszorg van Vlaanderen en deze van Wallonië. Serumstalen van 3170 schapen werden getest voor *T. gondii* antilichamen in een totaal lysaat antigeen (TLA) enzyme-linked immunosorbent assay (ELISA). Bijna 2400 (87,4%) stalen werden positief bevonden voor *T. gondii*. Antwerpen bezat de laagste seroprevalentie (65,2%), gevolgd door Wallonië (68,6%), Oost-Vlaanderen (96,7%), West-Vlaanderen (96,8%) en Limburg (97,3%). Vlaams-Brabant noteerde de hoogste seroprevalentie (97,8%). Enkel 8 van de 209 geteste schapenkuddes waren seronegatief. De seroprevalentie in Antwerpen en Wallonië was significant lager dan deze in andere provincies ( $P < 0.05$ ). Dit is de eerste seroprevalentiestudie in België. De hoge prevalentie toont aan dat er meer aandacht moet besteed worden aan schapenvlees als mogelijke bron voor humane toxoplasmosis.

In **hoofdstuk 4** worden de parasitaire distributie en de immuunrespons tijdens de acute fase van infectie bestudeerd. De schapen werden oraal geïnfecteerd met 3000 weefselcysten van de *T. gondii* PRU stam. Zowel de aanwezigheid van parasitair DNA als de parasiet-geïnduceerde interferon-gamma en interleukine-12 respons werden op 4, 8, 10, 14 en 21 dagen na infectie (dpi) onderzocht in de dunne darmmucosa, en de drainerende mesenteriale lymfeknopen, de popliteus lymfeknoop, de milt en leukocyten. Verder werd het verschijnen van *T. gondii*-specifieke antilichamen in serum dagdagelijks opgevolgd met behulp van een indirecte immunofluorescentietest voor IgM en IgG en met behulp van TLA-specifieke ELISA's voor IgG, IgG1, IgG2 en IgA. De resultaten tonen aan dat tachyzoieten reeds de eerste dagen na infectie de mucosa van het craniaal deel van de dunne darm binnendringen, terwijl de schapen nog steeds serologisch negatief zijn. Tegelijkertijd wordt

een hoge IFN- $\gamma$  respons en IL-12 respons geïnduceerd, en dit voornamelijk in de mesenteriale lymfeknopen. De opkomst van IgG1, op 8dpi, en IgG2, op 11 dpi, gaat gepaard met een afname, of zelfs verdwijnen van de IFN- $\gamma$  en IL-12 respons in de Peyerse Platen, de perifere bloed mononucleaire cellen (PBMC) en de popliteus lymfeknoop. Ondertussen kon *T. gondii* DNA worden gedetecteerd in de meeste mucosale en systemische weefsels. Het kon echter drie weken na infectie niet meer teruggevonden worden in de dunne darm, de popliteus lymfeknoop, perifere bloed mononucleaire cellen en de milt, wat erop zou kunnen wijzen dat de parasiet op dat moment deze weefsels verlaten had of eruit verwijderd werd door de immuunrespons. We veronderstellen dat IFN-gamma en IL-12 beide een rol spelen bij het controleren van de infectie.

In **hoofdstuk 5** volgden we de expressie van IFN-gamma (T-helper 1 cellen), IL-4 (Th2-cellen) en IL-10 mRNA (Treg cellen) door bloed mononucleaire cellen en de antilichaamrespons in serum tegen *T. gondii* totaal lysaat, *T. gondii* recombinant GRA1, rGRA7, rMIC3 en rEC2 in schapen gedurende de eerste 2 maanden na een *T. gondii* infectie. De immuunrespons in schapen werd vergeleken met de respons bij het varken om na te gaan of er species verschillen optraden en om te zien of dat dit de infectie kon beïnvloeden werd parasitaire load in het hart, de hersenen en 2 skeletspieren van schapen bepaald in vergelijking met deze in varkens. Hoewel de parasitaire load in schapen en varkens vergelijkbaar was, zagen we toch een duidelijk verschil in de antilichaam- en cytokine respons. Bij de schapen trad er een antilichaamrespons op tegen alle geteste *T. gondii* antigenen, maar voornamelijk tegenover rGRA7, rMIC3 en TLA terwijl bij de varkens enkel rGRA7-specifieke antilichamen konden worden aangetoond. Ook de cytokine respons leek meer uitgesproken bij schapen dan bij varkens. Zowel bij schapen als bij varkens trad er een IFN-gamma respons op al leek deze bij schapen vroeger op te treden en langer te blijven dan bij varkens. Ook de IL-10 en IL-4 mRNA expressie vertoonde in schapen een toename, maar later dan de IFN-gamma expressie en met meer variatie. In varkens werd echter geen toename gezien.

Wat betreft de diagnose van *T. gondii* suggeren de resultaten dat rGRA7-specifieke serumantilichamen bij levende schapen en varkens en bio-assay en qPCR op hartspierweefsel van geslachte dieren de beste testen zijn om besmetting met *T. gondii* te detecteren.

**Hoofdstuk 6** bevat de algemene discussie en de belangrijkste conclusies. In dit proefschrift werd voor het eerst de *T. gondii* seroprevalentie in de Belgische schapenhouderij bestudeerd. Echter dienen toch enkel kritische beschouwingen in acht genomen te worden omtrent de methodes gebruikt om het schapenserum te testen. De gebruikte immuunfluorescentie antilichaamtest (IFA) is niet gevalideerd noch gestandaardiseerd voor schapen. De TLA-ELISA, die werd aangewend, is een in-huis ontwikkelde test en dient verder geëvalueerd te worden om in de toekomst als screeningstest te kunnen gebruikt worden. Desalniettemin benadrukt de hoge seroprevalentie van *T. gondii* in schapen de noodzaak tot het uitvoeren van risico-analyses op Belgisch schapenhouderijen met verschillende managementsystemen, om al zo maatregelen te kunnen nemen ter controle van de infectie.

Vervolgens werd in dit proefschrift aangetoond dat, tijdens de acute infectie, hoge interferon-gamma responsen en lagere interleukine-12 responsen optraden. Tegelijkertijd was er sporadisch parasitair DNA detecteerbaar in de dunne darm, voornamelijk in het craniale deel. Vervolgens ging *T. gondii* zich meer spreiden in de darm en naar de systemische lymfoïde weefsels, dit tot twee weken na infectie, waarna bijna geen parasitair DNA meer kon worden aangetoond in de intestinale weefsels. Dit zou er kunnen op wijzen dat *T. gondii* de darm verlaat tijdens de acute fase van infectie om zich te spreiden naar meer systemische weefsels tijdens de subacute fase en dat de gastheer-immuunrespons niet in staat is deze verspreiding te stoppen noch te vrijwaren van parasieten. Inderdaad, tijdens de daaropvolgende subacute fase van infectie konden niet alleen hoge IFN-gamma worden waargenomen maar vertoonden ook IL-10 and IL-4 mRNA de neiging te stijgen, terwijl parasitair DNA werd aangetoond in het hart, de hersenen en skeletspieren. Ook bij varkens werden er in dezelfde weefsels, vergelijkbare hoeveelheden parasitair DNA aangetoond tijdens de subacute fase van de infectie en nam de IFN-gamma respons toe, maar iets later en minder lang dan bij het schaap. Echter IL-10 en IL-4 mRNA stegen niet.

Bij het evalueren van deze resultaten dient toch met enkele bemerkingen rekening gehouden te worden. Ten eerste, de hoeveelheid cytokines, vrijgesteld in het supernatans van de celcultuur, gedetecteerd door de cytokine ELISA zijn niet 100% vergelijkbaar met de mRNA niveaus gedetecteerd in real-time PCR. Daarenboven zou het nuttig zijn uit te breiden naar technieken gericht op het identificeren van cellen verantwoordelijk voor de cytokineproductie. Ten tweede, is het belangrijk te weten dat een negatief resultaat in PCR niet noodzakelijk een garantie biedt op parasitair vrij weefsel. Een PCR negatief resultaat kan te wijten zijn aan een ongelijkmatige verdeling van de parasiet over een weefsel omdat voor

PCR slecht zeer kleine hoeveelheden weefsel nodig zijn. Ten derde is het noodzakelijk te benadrukken dat er geen gevalideerde, noch gestandaardiseerde serologische testen voor handen zijn om toxoplasmosis in schapen te diagnosticeren. Ten vierde, aangezien schapen en varkens geïnfecteerd werden met verschillende type II *T. gondii* stammen, konden we niet aantonen dat de bekomen verschillen in immuunrespons te wijten zijn aan de gastheer of aan de stam.

Er is nog verder onderzoek nodig om de immuunrespons tijdens acute en chronische toxoplasmosis bij schapen volledig op te helderen en om diagnostische testen voor detectie van *T. gondii* op levende en geslachte dieren verder op punt te stellen.



## **CURRICULUM VITAE**





Delfien Verhelst werd geboren op 12 maart 1981 in Gent. Zij behaalde haar diploma secundair onderwijs aan Humaniora Nieuwen Bosch en startte hierna de studies Diergeneeskunde aan de universiteit Gent. In 2007 behaalde ze het diploma dierenarts met onderscheiding. Geboeid door het wetenschappelijk onderzoek, startte zij na haar studie, in 2007, een onderzoek voor de federale overheid bij de vakgroep Immunologie aan de Faculteit Diergeneeskunde van de Universiteit Gent. Waarna ze aansluitend een doctoraatsonderzoek, gefinancierd door het IWT, startte. Tevens behaalde zij recent het getuigschrift van de Doctoral School of Life Sciences and Medicine van de Universiteit Gent. Delfien Verhelst is auteur of mede-auteur van wetenschappelijke publicaties in nationale internationale tijdschriften. Zij was spreker en nam de voorbije jaren actief deel aan meerdere nationale en internationale congressen.

**Verhelst D.**, De Craeye S., Jennes M., Dorny P., Goddeeris B., Cox E., 2014. Interferon gamma expression and infectivity of *Toxoplasma* infected tissues from experimentally infected sheep in comparison with pigs. Submitted

**Verhelst D.**, De Craeye S., Dorny P., Goddeeris B. and Cox E., 2014. Parasite distribution and associated immune response during the acute phase of *Toxoplasma gondii* infection in sheep. Submitted

**Verhelst D.**, De Craeye S., Vanrobays M., Czaplicki G., Dorny P., and Cox E. Seroprevalence of *Toxoplasma gondii* in domestic sheep in Belgium. *Vet Parasitol.* 205: 57-61.

**Verhelst D.**, De Craeye S., Dorny P., Melkebeek V., Goddeeris B., Cox E. and Jongert E. 2011. IFN- $\gamma$  expression and infectivity of *Toxoplasma* infected tissues are associated with an antibody response against GRA7 in experimentally infected pigs. *Vet Parasitol.* 179:14-21.

**Verhelst D.**, Jongert E., Abady M., Petersen E. and Gargano N. 2008. Protective Th1 immune responses against chronic toxoplasmosis induced by a protein-protein vaccine combination but not by its DNA-protein counterpart. *Vaccine.* 26(41): 5289-95

Jongert E., Melkebeek V., De Craeye S., Dewit J., **Verhelst D.** and Cox E. 2008. An enhanced GRA1-GRA7 cocktail DNA vaccine primes anti-*Toxoplasma* immune responses in pigs. *Vaccine*, 26(8): 1025-1031

M. Jennes, S. De Craeye, **D. Verhelst**, P. Dorny, K. Dierick, V. Melkebeek, E. Cox. The consecutive infection with *Toxoplasma gondii* strains can affect the parasitic load in tissues of experimentally infected pigs. European Symposium on Porcine Health Management, Bruges, Belgium, 24-27/04/2012.

S. De Craeye, M. Jennes, **D. Verhelst**, P. Dorny, K. Dierick, V. Melkebeek, E. Cox. *T. gondii* strains and their dosage influence the parasitic load in tissues of experimentally infected pigs. Congress – 11<sup>th</sup> International Congress On Toxoplasmosis. Ottawa, Canada, 25-29/06/2011.

**D Verhelst**, S. De Craeye, Vesna Melkebeek and E Cox. Evaluation of parasite antigens in ELISA for the detection of *Toxoplasma* infection in pigs. Congress - IVth International Congress on Congenital Toxoplasmosis (ICOCT). Marseille, France 28-30/10/2011.

M. Jennes, S. De Craeye, **D. Verhelst**, P. Dorny, K. Dierick, V. Melkebeek, E. Cox. Cytokine responses in pigs infected with different dosages of *Toxoplasma gondii*. 9th International Veterinary Immunology Symposium. Tokyo, 16-20/08/2010.

**D. Verhelst**. Topics in parasitaire zoönosen, Toxoplasmose: voorkomen bij dieren en risico's voor de mens. Oral presentation IPV, Veterinaire Volksgezondheid en Zoönosen, 24/09/2009.

**D. Verhelst**, S. De Craeye, R. Mortier, M. Soen, P. Dorny and E. Cox. Toxoplasma: risks and prevention. Oral presentation at the Society for Applied Microbiology Summer conference: Fur, feather and fever - zoonotic challenges of the 21st century. Manchester, England, 6-9/07/2009.

**D. Verhelst**, S. De Craeye, M. Abady, E. Cox and E. Jongert. Correlation between serology and parasitic load in tissues of *Toxoplasma* infected pigs. Congress – 10<sup>th</sup> International Congress on Toxoplasmosis. Kerkrade, The Netherlands, 19-23/06/2009.

**D. Verhelst**, V. De Vriendt, S. De Craeye, E. Cox and E. Jongert. Screening of parasite antigens for the development of *Toxoplasma* ELISA in pigs and sheep, IPVS, Ghent, Belgium, 2009.

E. Jongert, V. Melkebeek, S. De Craeye, **D. Verhelst** and E. Cox. The GRA1-GRA7 cocktail DNA vaccine: protective cellular immune responses from mice to pigs. Oral presentation at the Forum on “*Critical Points in Vaccine Development*”, Toxoplasma centennial congress: from discovery to public health management. Armação dos Búzios, Rio de Janeiro, Brazil, 21–24/09/2008.

E. Jongert, **D. Verhelst**, M. Abady, E. Petersen and N. Gargano. Protective immunity against chronic toxoplasmosis induced with a protein-protein vaccine combination but not by its DNA-protein counterpart. Toxoplasma centennial congress. Armação dos Búzios, Rio de Janeiro, Brazil, 21 – 24/09/2008.

**D. Verhelst**, V. De Vriendt, S. De Craeye, M. Abady, E. Cox and E. Jongert. Evaluation of parasite antigens in ELISA for the detection of *Toxoplasma* infection in pigs. Toxoplasma centennial congress. Armação dos Búzios, Rio de Janeiro, Brazil, 21 – 24/09/2008.

E. Jongert, M. Abady, **D. Verhelst**, S. De Craeye, E. Petersen and N. Gargano. Evaluation of EC2 - GRA7 vaccine mixtures for the enhancement of protective immunity against infection with *Toxoplasma gondii*. Vaccine Conference – Celebrating 25 years of publication. Amsterdam, The Netherlands, 9-11/12/2007.

## **DANKWOORD**



*Regrets, I've had a few,  
But then again, too few to mention,  
I did, what I had to do:  
I did it my way!*

Aan het einde van een periode in je leven, is het toch altijd leuk om even je tijd te nemen, even terug te kijken op kleine successen en grote mislukkingen, alvorens met een tevreden gevoel een nieuwe start te nemen. Een van die kleine dingen die ik in de voorbije jaren heb opgestoken, is dat vele 'lezers' van een werk als dit niet verder raken dan deze pagina. Daarom houd ik er me aan om toch ook wat tijd te spenderen aan dit stukje. Wie had lang geleden ooit verwacht dat ik vandaag dit stukje zou schrijven? Ik niet in het minst: ik was immers niet meteen de allergrootste uitblinker als het over studieresultaten ging, misschien ook niet altijd even geconcentreerd, dus doctoreren was voor mij niet meteen een optie die er lang op voorhand zat aan te komen. Daarom, en dat durf ik toch wel toegeven, ben ik best een beetje trots, blij, gelukkig en zelfs vereerd dat ik vandaag dit laatste stukje tekst mag schrijven. Nog eenmaal krijg ik hier de kans om mijn verhaal te doen.

Je schrijft dit boekje niet alleen en je haalt geen doctoraat zonder begeleiding van mensen met ervaring in de wetenschapswereld. Het is rechtstreeks en onrechtstreeks, gewild en ongewild het werk van een hele hoop mensen. Een aantal van hen wil ik dan ook persoonlijk bedanken.

Ik wens hierbij dan ook mijn promotoren Prof Dr. Eric Cox en Prof Dr. Pierre Dorny te bedanken voor de kans die zij mij gaven! Ook Dr. Stéphane De Craeye wil ik hier bedanken voor zijn engelengeduld en de vele uren die hij in mijn experimenten stak! Uren heb ik in Brussel gesleten voor PCR's en IFA's, uren heb je aan mij gespendeerd om de technieken aan te leren en inzicht te krijgen in resultaten. Jouw hulp was onmisbaar bij het vervolledigen van dit proefschrift. Ook hier: Bedankt Stef!

Vervolgens zou ik ook alle leden van de lees en examen commissie willen bedanken voor het, met een kritisch oog, nalezen van dit werk en de constructieve opmerkingen.

Veel dank en respect gaat uit naar de mensen waarmee je de werkvloer en de gekende ups-and-downs deelt, mijn mede-immunologen en Hermanologen.

Bureaugenoten, jullie hebben de laatste loodjes van heel dichtbij meegemaakt toch hebben we samen heeeel toffe kletsmomenten gehad en gezellige avonden. Thary en Korneel, jullie zijn diegenen die volgen, ik kan jullie nu enkel succes wensen met het schrijven van jullie

doctoraat! Kim nog heel veel chance met uw onderzoek en natuurlijk ook met het koken ☺. Christophe ook jij heel veel succes met je doctoraat.

Bert, respect voor jouw kennis, voor jouw ochtendhumeur daarentegen... Gosia, gaandeweg werd je partner in crime, jou wens ik het beste! Ut, you are the next one, success with your doctorate!

Joanna and Evelien, the “calve colleagues” Maria, Elisa, Yu, Céline, Jochen, Cliff, Manon en Herman, het was fijn met jullie het verdiep te delen.

Rudy, Griet, Hanne, Simon jullie zijn van onschatbare waarde voor het labo. Rudy, bedankt voor al jouw hulp met de schapen en de varkens. Ik apprecieer dat ik steeds op jouw hulp kon rekenen bij de slachtingen in de vroege uurtjes en bij alle staalnames. Griet, Hanne, Simon: bedankt voor de ELISA's, de helpende handen bij de slacht, het maken van weefselcoupes, de sfeer en zoveel meer....

Als ik terugkijk op de afgelopen jaren zijn er nog een aantal mensen die ik niet mag vergeten. Erik, Vesna, Annelies, Michaëla, Bakr, Edith, Eva, Maryam, Philippe, Kris, Nina, Pieter, Tine, Maaike, Rienske, Anastasia, Marjolein, Denise bedankt voor jullie raad en daad!

Sarah, Ann en Gert, bedankt voor de administratieve beslommeringen. Ann, de boekhoudende steunpilaar, maar voor mij was en ben je nog véél meer dan dat .... Echt onmisbaar!! Mieke, ISPC maatje, aan jouw organisatorisch talent kon niemand raken. Je deed alles om het telkens opnieuw volledig naar ons zinnen geregeld te krijgen. Dank je voor al mijn administratieve zorgen op te lossen, we missen je! Sarah ook jij bedankt voor al het organisatorische en administratieve werk om mijn doctoraat in orde te krijgen. Charlotte bedankt om alle proefversies tot de laatste letter te willen verbeteren op taalfouten; er staat nog een dessertbuffet op jou te wachten! Professor Van Broekhoven, ook U zou ik willen bedanken voor de constructieve aanpassingen. Dirk op het werk, Matthias naast het werk, dank je om de combinatie van een panikerende ik en crashende computers altijd terug tot een geruststellende samenwerking te kunnen brengen.

Mona, Isabelle, Maité, Alex, merci de m'avoir accueilli si cordialement à Pasteur ou WIV de Bruxelles. Les sept années que j'ai passé chez vous m'ont appris plein de choses, non seulement scientifiques mais aussi personnelles. Tous les thesards que j'ai eu l'honneur de rencontrer: c'est grâce à vous que j'ai vécu un agréable séjour. Mona et Isabel merci pour la patience et les explications que tu m'as données sur l'IFA et sur le PCR. C'était toujours amusant de passer au bureau 106 et 110!

Jurgen ook jij bedankt voor de toffe dagen in Brussel gedurende de laatst maanden!



Dit is echter ook het resultaat van de steun die ik al vele jaren krijg van mijn familie. Ik weet dat het melig klinkt maar dit is echt het moment om het te zeggen: mama, papa, zus bedankt om er te zijn voor mij. Mama, papa, ik weet niet waar te beginnen. Jullie hebben van mij gemaakt wie ik ben en mij alle kansen gegeven en anderzijds, even belangrijk, van mij gemaakt wie ik *niet* ben. Ook al was het laatste jaar en vooral de laatste maanden zeer zwaar voor jullie, dit proefwerk was absoluut niet ontstaan zonder jullie extra oppeppende woorden. Mama, niemand had een aantal maanden geleden gedacht dat je dit kon bijwonen, maar nu ben ik blij dat ik, ondanks alles en met veel steun, jouw wens heb kunnen waar maken! Dit is dan ook deels van en voor jullie! Zus, je bent zo anders en toch ook vaak schokkend hetzelfde! Johan en Chris, ook jullie wil ik bedanken voor het immer paraat staan voor ons gezin zeker in de afgelopen maanden. Het heeft de combinatie gezin/doctoren gemakkelijker gemaakt.

Mijn familie en vrienden: Pedro, Amelie, Gilles, Mieke, Wim, Els, Peter, Sarah, Cédric, Ann, Karel bedankt om me op te lappen, te bellen en me op het einde ook af en toe eens uit mijn huis te komen halen om eens samen op stap te gaan. Allemaal heel erg bedankt!

Tenslotte komt het dankje voor de belangrijkste personen. Aurélie en Maïté, jullie beseffen het waarschijnlijk nog niet, maar jullie hebben mijn ganse leven in positieve zin op zijn kop gezet. Jullie lach en knuffels hielpen om het toch vol te houden en jullie hebben er altijd mee voor gezorgd dat ik mij nooit oversliep. Een knuffel en dikke duim van de mama.

Bart, jouw optimisme was grenzeloos, net als je vertrouwen in mijn kunnen. Jij hebt als geen ander met me meegedeeld gedurende mijn doctoraat, ook al snapte je er naar het einde toe als maar minder van. Ik besef ten volle dat ik op bepaalde momenten niet de makkelijkste persoon ben geweest om mee samen te leven en ik weet dat jij net zo blij bent als ik, dat dit erop zit! Samen hebben we onze opleiding, de verbouwingen en nu mijn doctoraat doorstaan. Hopelijk breken er nu iets rustigere tijden aan waarin we wat meer van ons gezin kunnen genieten, zonder aan een project bezig te zijn. Bedankt voor je onvoorwaardelijke steun gedurende de voorbije 13 jaar, laat de rest maar volgen! Bedankt Bolle!

***“Science seldom proceeds in the straight forward logical manner imagined by outsiders. Instead, its steps forward (and sometimes backward) are often very human events in which personalities and traditions play major roles.”***

***(James Watson)***